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The Effects of the Mutator Gene on Molecular Changes and Mutation in *Salmonella typhimurium*†

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(*Received 19 May 1960*)

Frequency of mutation was followed in histidine-requiring mutants of the LT2 and LT7 strains of *Salmonella typhimurium*. Mutagenic agents used were: 2-aminopurine (AP), 5-bromodeoxyuridine (BD), sodium nitrite (NO_2), *t*-butyl hydroperoxide (*t*-butyl) and *beta*-propiolactone (BPL). Some of the histidine mutants were of spontaneous origin, while others were induced by ultraviolet irradiation (u.v.) and 2-aminopurine.

All but one of the twelve auxotrophic derivatives of the LT2 strain, which were originally obtained after treatment with AP, showed an increase in "reverse" mutation frequency with BD. The u.v.-induced auxotrophs gave a variety of responses with the different mutagenic agents and no particular mutational pattern predominated.

Of the 54 LT2 strains of spontaneous origin tested, 26% were affected by base analogues, 45% by BPL and 28% by *t*-butyl. In 52% of the cases, *t*-butyl and BPL were mutagenic for the same strains.

A greater proportion of the LT7 strains of spontaneous origin responded with base analogues and the other chemicals. The increase paralleled the presence of the mutator gene. All chemicals used, except nitrite, were mutagenic with 90% or more of the 80 LT7 strains possessing the mutator gene. The pattern of induction to prototrophy in LT7 strains lacking mutator was very similar to that of LT2 strains.

1. Introduction

Recent studies with bacteriophage by Benzer & Freese (1958) and Freese (1959), and with tobacco mosaic virus by Schuster & Schramm (1958) have enabled these investigators to postulate a molecular basis for mutation. In each case the final change or mutation was accomplished by the presence of an analogue of the purines or pyrimidines in the genetic material, nucleic acids (Watson & Crick, 1953). It was assumed that the analogues paired during replication with a base other than the one originally present. The new base would then pair with its normal complement, thereby changing one base pair of the double-stranded DNA molecule. If during this process a purine is substituted for another purine, and a pyrimidine for a pyrimidine, the change is referred to as a "transition" mutation (Freese, 1959). In contrast, a "transversion" mutation consists of the substitution of a purine for a pyrimidine, etc. The latter type of alteration is not considered to be effected by the presence of the base analogues used to date.

Comparison of the effects of base analogues with other mutagens should give an insight into the type or types of mutations they may cause. Also, by using mutants of

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spontaneous origin, the different types of mutation obtained under "normal" conditions of growth may be discerned. Studies on the effect of base analogues and other chemical mutagens were made on spontaneous mutants of the LT2 and LT7 strains of *Salmonella typhimurium*.

2. Materials and Methods

Histidine mutants of spontaneous origin from *S. typhimurium*, strains LT2 and LT7, were used. In addition, LT2 histidine mutants induced by ultraviolet (u.v.) and by 2-aminopurine (AP) were used. The origins of many of the mutants are listed in Table 1 of Hartman, Loper & Serman (1960) and the locus designation of these workers will be followed here. All strains were maintained on stock agar slants at 4°C. Transfers of these stocks into nutrient broth were incubated at 37°C with aeration overnight and used for testing the mutagenicity of the various chemical agents.

The minimal medium used to assay the mutation from auxotrophy to prototrophy was the following: 1·5 g KH_2PO_4; 0·1 g $MgSO_4$; 1 g NH_4Cl; 3·5 g Na_2HPO_4; 9 g lactic acid; 15 g BBL agar (from Baltimore Biological Lab.); and 1000 ml. water. The medium was adjusted to pH 7·0 with 1 N-NaOH and enriched with 1·25% nutrient broth. The enrichment supported growth to a final population of about $1{\cdot}5 \times 10^9$ auxotrophs per plate.

To test the effects on mutation frequencies of various chemicals, the paper disc method described by Iyer & Szybalski (1958) was employed. After spreading 0·05 ml. of a saturated culture, about 5×10^7 bacteria per plate, a sterile paper disc was placed in the center of each plate. The amount of each chemical used per disc was: 0·05 ml. of 0·1 dilution of *beta*-propiolactone (BPL), 0·01 dilution of *t*-butyl hydroperoxide (*t*-butyl), 1 mg/ml. 2-aminopurine (AP) or 5-bromodeoxyuridine (BD) and 0·03 ml. of 0·1 M-$NaNO_2$ (NO_2). All tests were run in triplicate.

After adding the various chemicals, the minimal plates were incubated at 37°C for 48 hr, the number of prototrophic colonies counted, reincubated 24 hr at 37°C and prototrophs counted again. Since there is a concentration gradient of mutagens on the plates, the change observed in the mutation frequencies as a result is considered semi-quantitative only and the treatment is scored strongly positive, positive, weakly positive, or negative (no effect).

When it was observed that a considerably higher percentage of LT7 strains, as compared to LT2, exhibited an increase in back mutation frequency with base analogues and the other chemical treatments, it was thought that the presence of the mutator gene might be responsible. Consequently, all of the LT7 stocks were tested for the presence of the mutator gene by the streptomycin technique described by Miyake (1960). A 0·001 ml. sample of each culture to be tested was spread over approximately one square inch of nutrient agar containing 100 μg/ml. of streptomycin. The plates were incubated at 37°C for 24 hr. Those strains exhibiting more than 100 colonies per square were scored as possessing the mutator gene, whereas those strains showing a few or no colonies on each square were scored as lacking mutator. Wild type LT2 (mutator absent) and an LT7 wild type strain found by Miyake to possess mutator served as controls.

To determine if the base analogues acted in bacteria as they do in bacteriophage, 12 LT2 strains induced with AP were tested with the chemical mutagens. Also strains originating after u.v. treatment were used to see if any specific response predominated in the back mutation tests. Controls were adequate to assure that these strains were definitely induced by the treatment.

3. Results

In the following Tables are summarized the results of the experiments in which mutagens were used. Included is the histidine sub-locus, strain of origin (LT2 or LT7), and relative mutation frequency of the control without mutagens added (high—indicates over 100 prototrophic colonies per plate; low—under 100 colonies).

Table 1 shows the results obtained with the LT2 strains. The incidence in which base analogues (either BD, AP, or both) increase the reversion frequencies for the

LT2 stock is 26% of the 54 strains tested. Mutation frequencies for most of these responsive alleles are stimulated by BD only, a few by AP and by BD, and one only by AP and by NO_2. BPL and *t*-butyl are more effective mutagenic agents not only increasing the mutation frequencies where base analogues are mutagenic but also

TABLE 1

The effect of chemical agents on increasing the reversion rate of spontaneous LT2 histidine mutants

Mutant	Rate	B	T	BD	AP	NO_2	Mutant	Rate	B	T	BD	AP	NO_2
D-23	low	+++	++	+	—	—	A-48, D-39, A-140	low	+	—	—	—	—
C-50, F-42	low	+++	+	+	—	—	D-130, H-32	low	+++	—	—	—	—
C-43, C-151	low	+	+	+	—	—	B-21, F-58	low	++	—	—	—	—
F-45	low	++	+	++	—	—	B-59, F-197	low	+	—	—	—	—
D-49	low	+++	+	—	+	+	B-61	low	—	+	—	—	—
A-33, F-148	low	—	—	+++	—	—	F-103	low	—	++	—	—	—
F-131, F-147	low	—	—	+	—	—	B-18, B-20, B-24	low	—	—	—	—	—
G-52	low	+	—	+	+	—	B-40, B-47, B-53	low	—	—	—	—	—
F-44, B-136	low	+	—	+	—	—	B-138, B-143, C-13	low	—	—	—	—	—
D-54	low	+	+	—	—	—	D-27, D-133, D-137	low	—	—	—	—	—
B-14	low	+++	++	—	—	—	D-149, D-150, D-60	low	—	—	—	—	—
D-142	low	++	++	—	—	—	C-129, E-35, E-145	low	—	—	—	—	—
B-102	high	+	+	—	—	—	E-162, D-139, F-132	low	—	—	—	—	—
C-31, G-46	low	+++	+	—	—	—	F-41, B-12	low	—	—	—	—	—

+++ Over tenfold increase in mutation frequency.
++ Five-to ninefold increase in mutation frequency.
+ Under fivefold increase in mutation frequency.

B = *beta*-propiolactone.
T = *t*-butyl hydroperoxide.

TABLE 2

The effect of chemical agents on increasing the reversion rate of spontaneous LT7 histidine mutants having the mutator gene

Mutant	Rate	B	T	BD	AP	NO_2	Mutant	Rate	B	T	BD	AP	NO_2
A-253, D-123, B-229	high	+	+	+	+	—	A-269, A-277, B-234	high	+	+	+	—	—
B-238, B-241, B-243	high	+	+	+	+	—	B-293, A-69, A-81	high	+	+	+	—	—
B-257, C-280, C-117	high	+	+	+	+	—	D-215, D-216, D-244	high	+	+	+	—	—
B-65, D-74, D-82	high	+	+	+	+	—	C-252, D-64, D-263	high	+	+	+	—	—
D-83, D-94, D-182	high	+	+	+	+	—	E-301, E-284, F-221	high	+	+	+	—	—
D-220, D-223, D-237	high	+	+	+	+	—	F-224, G-265, F-240	high	+	+	+	—	—
D-248, D-254, D-68	high	+	+	+	+	—	G-72, F-251, D-92	low	+	+	+	—	—
E-264, E-266, F-76	high	+	+	+	+	—	D-90, D-78, D-66	low	+	+	+	—	—
F-93, F-235	high	+	+	+	+	—	C-151, C-169	low	++	++	++	—	—
F-227, F-228	low	+	+	+	+	—	C-212, E-242	high	+	—	+	—	—
E-164, G-119	low	+++	+	—	+	+	D-85, D-230	high	—	—	—	—	—
A-231, C-258, E-250	high	—	+	+	—	—	A-179, D-84	high	++	+	+	—	—
E-259	high	—	+	+	—	—	A-80	high	+++	+++	+	—	—
A-294	low	++	++	+	+	—	D-79	low	+	+	+	—	—
C-121	high	++	+	+	+	—	C-225	low	+	+	+++	—	—
F-73	high	+++	++	+++	+	—	F-256	low	—	+	—	—	—
C-261	high	++	++	++	—	—	D-171, D-245	high	+	+	++	—	—
C-86	high	+	+	—	+	—	D-88	high	+	—	+	+	—
D-170	high	+	—	++	—	—	F-219	low	+	+	++	—	—

in those strains unaffected by BD and AP treatments. The spectrum of effectiveness of mutagenicity of BPL and *t*-butyl overlaps in many instances: 52% of the mutants which respond to one of the agents also respond to the other.

LT7 strains show an induced mutational pattern varying greatly from that noted above for the LT2 strains. A considerably higher proportion of strains were induced to revert to prototrophy by all chemical treatments used. Mutator was found to be present in many of the LT7 strains. From Table 2 it may be seen that practically all

strains having mutator showed positive results with the two base analogues and with *t*-butyl and BPL. An increase in the reversion frequency was observed with 78 of the 80 strains of LT7 with mutator when treated with AP, BD or both individually, 71 of 80 with *t*-butyl and 73 of 80 with BPL.

Table 3 shows that LT7 strains lacking mutator reacted very similarly to the LT2

TABLE 3

The effect of chemical agents on increasing the reversion rate of spontaneous LT7 histidine mutants without the mutator gene

Mutant	Rate	B	T	BD	AP	NO_2	Mutant	Rate	B	T	BD	AP	NO_2
A-100	low	++	+	—	+	+	A-97	low	—	—	+++	—	—
A-300	low	+	+	—	+	+	C-28	low	—	—	+	—	—
D-124	low	+++	++	—	+	+	A-106	low	+++	—	—	—	—
B-262, A-67, A-153	low	+	+	—	—	—	A-157, C-110, D-77	low	+	—	—	—	—
A-263	low	+	+	+	—	—	A-159	low	++	—	—	—	—
F-91	high	+	+	+	—	—	D-108, D-127	low	—	++ +	—	—	—
A-30	low	++	++	—	—	—	A-89, A-246, B-29	low	—	—	—	—	—
B-116, D-113, E-105	low	+++	+	—	—	—	B-118, D-27, C-104	low	—	—	—	—	—
B-167, C-115, G-70	low	+	+	—	—	—	C-120, C-122, D-123	low	—	—	—	—	—
-161	low	+	+	—	—	—	D-154, D-160, E-99	low	—	—	—	—	—
D-112	low	+++	+++	—	—	—	E-125, F-109, H-107	low	—	—	—	—	—
D-166	low	+++	++	—	—	—							

strains. The base analogues increased the reversion frequency in 9 of the 42 strains tested (21%), BPL in 23 of 42 (54%), and *t*-butyl in 20 of 42 (49%) tested. A high degree of overlapping in the effective spectra of the latter two treatments was observed once more. Also, treatment of these strains with *t*-butyl and BPL often increased reversion frequencies whether the two base analogues were effective or not.

The mutation to streptomycin resistance in LT7 derivatives having the mutator gene is generally increased 100- to 1000-fold over that of the LT2 strains or the LT7 strains not having this gene. Two strains of LT7 which were affected by AP and NO_2, but not by BD, exhibited toward streptomycin resistance mutation frequencies much lower than those of most other LT7 strains having mutator; these strains showed an

TABLE 4

The effect of chemical mutagens on u.v.-induced LT2 auxotrophs

Mutant	Rate	B	T	BD	AP	NO_2	Mutant	Rate	B	T	BD	AP	NO_2
600	low	+++	++	+	+	—	603	high	++	++	+	+	—
606	low	+++	+++	—	—	—	11	low	+++	++	—	—	—
602	low	++	++	—	—	—	35	low	+	+	—	—	—
601	low	—	++	—	—	—	604, 605, 607	low	—	—	—	—	—

increase from two- to tenfold. Three LT7 strains in which AP and NO_2 were effective gave doubtful positive results with the streptomycin technique for the mutator gene. These exceptional LT7 strains are being further investigated to determine if they contain an altered mutator gene or a mutator gene different in location from that of the other LT7 stocks.

Several mutants listed in the first three tables are inseparable as to locus on the basis of complementation tests (Hartman *et al.*, 1960), although they may vary in

TABLE 5

Comparison of the effectiveness of u.v. with other mutagens on spontaneous mutants

Mutant	u.v.†	B	T	BD	AP	NO_2	Mutant	u.v.†	B	T	BD	AP	NO_2
C-117	+++	+	+	+	+	—	C-146, C-28	—	—	—	+	—	—
C-121	+++	++	+	+	+	—	A-33, F-148	—	—	—	+++	—	—
A-100	+++	++	+	—	+	+	B-21	—	++	—	—	—	—
D-113	+	+++	+	—	—	—	H-32	—	+++	—	—	—	—
D-124	+	+++	++	—	+	+	F-103	—	—	++	—	—	—
C-115	++	+	+	—	—	—	F-131	—	—	+	—	—	—
B-116	+++	+++	+	—	—	—	D-112	—	+++	+++	—	—	—
C-110	+++	+	—	—	—	—	A-140, B-12, B-20	—	—	—	—	—	—
A-106	+++	+++	—	—	—	—	B-24, B-138, E-145	—	—	—	—	—	—
D-130, C-120	+++	—	—	—	—	—	B-143, D-27, F-132	—	—	—	—	—	—
A-97	++	—	—	+++	—	—	D-133, D-137, D-149	—	—	—	—	—	—
D-18	++	—	—	—	—	—	C-122, B-118, C-129	—	—	—	—	—	—
B-29	+	—	—	—	—	—							

† Data of M. Demerec, A. Chou & P. E. Hartman (personal communication).

TABLE 6

The effect of chemical mutagens on 2-aminopurine-induced LT2 auxotrophs

Mutant	Rate	B	T	BD	AP	NO_2	Mutant	Rate	B	T	BD	AP	NO_2
443, 446	low	++	+	+	+	—	441	low	++	—	+	—	—
440	low	+++	+	++	—	—	444	low	+	—	+	—	—
421	low	++	+	+	—	—	420	low	—	+	+	—	—
422	high	++	+	+	—	—	447, 430	low	—	—	+++	—	—
427	low	—	+	+	++	—	428	low	+	+	—	—	—

TABLE 7

Summary of mutagenic responses of the strains tested

No. tested:	54	42	80	12	10	Induced mutation pattern				
Strain:	LT2	LT7	LT7†	LT2	LT2					
Origin:	Spont.	Spont.	Spont.	AP	u.v.	B	T	BD	AP	NO_2
	41	36	3	0	30	—	—	—	—	—
	17	12	0	0	0	+	—	—	—	—
	4	5	1	0	10	—	+	—	—	—
per cent	11	31	0	8	40	+	+	—	—	—
with	9	5	0	17	0	—	—	+	—	—
pattern	0	0	5	8	0	—	+	+	—	—
(nearest	4	0	4	17	0	+	—	+	—	—
per cent)	0	0	1	0	0	+	+	—	+	—
	0	0	0	8	0	—	+	+	+	—
	2	0	1	0	0	+	—	+	+	—
	11	5	43	25	0	+	+	+	—	—
	0	0	41	17	20	+	+	+	+	—
	4	7	3	0	0	+	+	—	+	+

† Mutator present.

other characteristics. Pairs such as *his*-12 and -24, *his*-40 and -47, and *his*-60 and -90 have identical mutation patterns as well as locus. On the other hand, *his*-143 and -65 give different patterns of mutation as do *his*-133, -78 and -54, although the locus appears to be the same.

The strains derived by u.v. treatment of LT2 show no striking common pattern of mutation (Table 4). A few strains were induced to mutate by the two analogues, *t*-butyl and BPL, some only with *t*-butyl or BPL, still others were not affected by any treatment (mutagen stable strains). Although the sample is small, the patterns of mutation do not overlap completely that of spontaneous mutation in LT2; for example, strains *his*-600 and 603 (Table 4) give mutational responses not found for any of the 54 spontaneous LT2 mutants (Table 1).

Table 5 compares, in spontaneously derived mutant strains, responses to u.v. with the other treatments used to induce mutation to prototrophy. As in the u.v.-induced auxotrophs, no specific type of change predominates. On the other hand, all but one of the 12 LT2 strains, induced to auxotrophy by AP, exhibited an increase in reversion frequency in the presence of BD (Table 6).

Table 7 summarizes the results obtained with the mutagenic agents. The similarities in response of LT2 and LT7 strains without mutator can readily be seen. The differences in response when mutator is present in LT7 or when AP-induced mutants are used in the test are also apparent.

4. Discussion

It is considered that the results are semi-quantitative in nature. The possibility remains that some positive results were missed due to the nature of the technique, inherent physiological differences, or differences in growth behaviour between mutants. The latter possibility is considered unlikely since all of the mutants have the same primary growth requirement. Also the prototrophs were not tested genetically to see if they represent true back mutation.

The increase by BD in the reversion frequency in all but one of the 12 LT2 strains induced by AP indicates a specific primary mechanism of mutation induction by each of these agents. This suggests that the modes of mutation induction by AP and BD in bacteria may be similar to those proposed by Freese (1959) for bacteriophage. That is, once the analogue is incorporated into the DNA, replacing the normal base, a base pair change is accomplished with a purine substituting for a purine (transition mutation). For example, BD may be incorporated into DNA in place of thymine. At the next replication, the BD pairs with guanine and a further replication enables guanine to pair with its normal partner, cytosine, thereby changing an A/T pair to a G/C pair. The reciprocal type of transition could be effected should BD pair with guanine. Similarly, AP can bring about the same types of changes as it has been proposed that AP can substitute for either of the normal purines. Consequently, one analogue has the ability to "reverse" mutations induced by the other analogue.

The use of spontaneous mutants derived from two different wild type strains of *Salmonella* gives a suggestion of what molecular changes " normally" give rise to spontaneous mutation. Since 26% of the LT2 mutants responded to base analogues, it is inferred that about a quarter of the single site mutations leading to the histidine-less phenotype in LT2 are of the transition type $\begin{matrix} A \rightarrow G \\ T \leftarrow C \end{matrix}$ (Freese, 1959). The susceptibility of these mutants to base analogues indicates that many of the single site

mutations are truely "point" mutations on the molecular level, i.e. involve changes in single base pairs in DNA. Those mutants in which the base analogues are ineffective, or which spontaneously mutate to prototrophy but are mutagen stable, may represent different changes; (1) of single base pairs representing various types of transversion mutations such as $\begin{matrix} A \rightarrow T \\ T \leftarrow A \end{matrix}$ etc. (Freese, 1959), or (2) alterations involving a greater DNA segment than single base pairs.

Mutagen stable characteristics have been studied previously by Demerec (1953) and Labrum (1953). Demerec found that these types of mutants were equally distributed throughout the genome, whereas Labrum working with streptomycin alleles found no mutagenic stable strains. The strains tested in these studies show that mutagenic stability is randomly distributed throughout the various sub-loci of the histidine gene, a result similar to that observed by Demerec. This is consistent with Demerec's proposal that mutagen stability is associated with certain alleles and not any one locus.

The capacities of BPL and *t*-butyl to increase the number of mutations in many instances where base analogues do, and in others where base analogues do not, indicate that they are capable of bringing about both transition mutations and other types of mutation. It is interesting to note the overlapping spectra of induced mutation by *t*-butyl and BPL. These chemicals have one characteristic in common; both are polymerizing agents.

The results of both forward (Table 4) and reverse (Table 5) mutations indicate that treatment with u.v. is nonspecific in that both presumed transitions and transversions are affected. The lack of a specific pattern is in agreement with the method of mutation proposed by Haas & Doudney (1957) for u.v. irradiation; namely, that u.v. causes changes *in vivo* of precursors of DNA and that these are intermediate in bringing about mutation of the transversion type. The transition type of mutation observed following u.v. may represent some direct effect upon DNA.

Mutator genes have been described in maize by McClintock (1951) and in *Drosophila* by Demerec (1937) as well as in bacteria; in *E. coli* by Treffers & Spinelli (1954) and in *Salmonella* by Demerec *et al.* (1957). The mutators of higher organisms appear to affect specific genes or loci. In bacteria, many loci, although not all, are affected by mutator genes. The mutator of *Salmonella* LT7 increases the mutation frequency in either direction, i.e. not only are auxotrophs more abundant in LT7 than in LT2 cultures but the LT7 auxotrophs back mutate at a much higher frequency. The characteristic of the LT7 strains possessing mutator to respond to base analogues in 95% of those tested, shows that this gene, in some way, facilitates an event leading to a transition. Possibly the LT7 mutator gene controls the production or overproduction of some compound(s) responsible for this behavior. As described in the text, several LT7 derivatives exhibit different levels of increase in reversion frequencies, as well as different patterns of mutagenicity. The possibilities exist that LT7 possesses not one, but two different mutator genes, or that two different alleles of a single locus are present in the various stocks. An answer to these questions is being sought by the determination of the position(s) of mutator(s) in each of these stocks.

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Comparative Studies of Fibroins

I. The Amino Acid Composition of Various Fibroins and its Significance in Relation to their Crystal Structure and Taxonomy

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The amino acid composition of 74 fibroins from various silk-producing arthropods has been determined, 26 of these fibroins being analysed quantitatively, and the remainder semi-quantitatively after paper chromatography. The results show that there are wide variations in the amino acid compositions of the fibroins, although all those examined so far are characterized by the high proportions of the simple amino acids, glycine, alanine, and serine, that they contain. No general correlation could be found between the biological classification of the arthropods and the amino acid composition of the fibroins they produce, though a few limited correlations were observed. In particular, the fibroins produced by the Saturniidae resemble one another in amino acid content and always contain more alanine than glycine. Some fibroins from biologically unrelated species have closely similar amino acid compositions and crystal structures. The significance of the results in relation to the crystal structure, taxonomy, and biological function of the proteins is discussed.

1. Introduction

The chemical constitution of the common silk fibroin of commerce, that of *Bombyx mori*, has been the subject of investigation for many years, and mean values of its amino acid composition, derived from many sources, are given by Lucas, Shaw & Smith (1958). By comparison, the fibroins of other Lepidoptera and silk-producing Arthropoda have received little attention, the few investigations having been mainly devoted to the so-called "wild silks," such as tussah, that have some commercial value.

Early work on the analysis of *Bombyx* and tussah silk fibroins, in which the names of Emil Fischer and Abderhalden predominate, has been summarized by Timon-David (1942–45). One analysis by Abderhalden (1922) of *Bombyx* silk fibroin shows a recovery of 86·4% of the protein as amino acids, and is a remarkable achievement when one considers the techniques that were then available. In general, however, analyses prior to 1930 show low recoveries of amino acids, in particular of serine. The range of fibroins that have been investigated has been slowly extended during recent years (see Lucas *et al.*, 1958), and further extension makes a definition of fibroins desirable. They are best defined as filaments produced by certain species of Insecta (insects) and Arachnida (spiders) from solutions of protein secreted in special glands in the arthropod. A number of different types of these glands are to be found within the Arthropoda. With the Lepidoptera, which are the most abundant producers of fibroin, the protein is synthesized in the silk glands that are regarded as modified salivary glands and debouch in the lip of the larva. The protein products of the collaterial glands of the Chrysopidae are drawn into filaments to serve as egg-stalks. With this family a fibroin is also produced by the larva to form a pupal cocoon.

With spiders a number of different types of silk gland have been observed; they occur even in the same species and doubtless give rise to the different kinds of silk produced by the organism to serve various functions ranging from protective cocoons (for the eggs) to nests and trapping webs.

In 1955 Lucas, Shaw & Smith published analyses of seven fibroins from species belonging to three families of Lepidoptera, and of one fibroin from a species of spider, and showed that there were considerable variations in their amino acid composition, especially between different families. Kirimura (1957), using methods of microbiological assay, examined the amino acid composition of the fibroins produced by nine species of silkworm from the Bombycidae, Thaumetopoeidae and Saturniidae families and attempted to classify them according to their amino acid distribution, especially in relation to the proportions of glycine and alanine. Similarly Duchâteau, Florkin & LeClerq (1960), also employing microbiological methods, have analysed a number of fibroins from Lepidoptera, mainly from the Saturniidae, and suggested that silk moths that are taxonomically related produce fibroins whose amino acid compositions are also related through a characteristically high proportion of certain amino acids, or groups of amino acids.

In the present paper, we give the results obtained from the amino acid analyses of over 70 different fibroins, and discuss the significance of these results in relation to the biological classification of the arthropods producing the fibroins and to the X-ray diffraction patterns. The X-ray studies have been undertaken by Warwicker of our laboratories, who in recent papers (Warwicker, 1956, 1960*a*, 1960*b*) gives results obtained from an examination of most of the fibroins that we have examined chemically. Warwicker shows that the classification of these fibroins according to their X-ray diagrams does not rigidly follow the biological classification. All the fibroins gave identical repeat distances in the direction of the fibre axis and of the hydrogen bonds, but they varied in dimensions in the direction of the side-chains, and could be grouped accordingly. Five such groups emerged, with mean side-chain dimensions varying from 9·3 Å for *Bombyx* silk fibroin to 15·7 Å for the fibroin of the egg cocoon of the spider *Nephila senegalensis*. A detailed account of Warwicker's work is being published at the same time as this paper (Warwicker, 1960*b*).

2. Experimental

(a) *Materials*

The species producing the fibroins examined by us are shown in Table 1 together with the form of the fibroin and an indication of the methods used to purify the samples.

The classification and nomenclature adopted is that of Kloet & Hincks (1945) for British species and Seitz (1906) for non-British species; and within any one family, the species are arranged in alphabetical order.

The fibroins investigated have been mainly in the form of cocoons, though several have been the nests which some caterpillars construct as communal resting places during the larval period. Where fibroin in the form of a nest and fibroin from the subsequent cocoons of the species have been available, they have been analysed and compared.

The *Anaphe* genus constructs a large communal cocoon in which the larvae then spin their own individual cocoons. In some species, for example *Anaphe infracta*, this communal cocoon may be as large as a coconut. Many of the Saturniidae construct substantial cocoons, those of the genus *Antheraea*, which furnish tussah silk, being large solid structures. Others, such as the cocoon of *Dictyoploca japonica*, are of open basket-like construction, with the chrysalis visible within.

The appearance, shape, size, and similar physical characteristics of the cocoons produced by various Lepidoptera vary within the same family but are usually rather similar in the

TABLE 1

Systematic list of fibroins investigated and semi-quantitative estimations of amino acid composition by paper chromatography

Family	Species	Form of fibroin	Method of Preparation	X-ray Group	Gly	Ala	Val	Leu/Ileu/Phe	Ser	Thr	Asp	Glu	Tyr	Pro	Arg	Lys	His
				CLASS: INSECTA													
Order: Neuroptera																	
Chrysopidae	*Chrysopa flava*	e	O	* 3a	5	4	1	1	6	1	1	1	1	0	1	1	0
Order: Lepidoptera																	
Arctiidae	*Arctia caja*	c	F	4	4	5	1	1	3	1	1	1	1	1	1	1	1
Cymbidae	*Bena prasinana*	c	A	1	5	4	1	1	3	1	1	1	1	0	1	1	0
Caradrinidae	*Apatele rumicis*	c	A	n.d.	4	3	1	1	3	1	1	1	1	1	2	1	0
Lymantriidae	*Euproctis chrysorrhoea*	c	O	3b	4	5	1	1	3	1	2	1	1	1	1	1	0
	Euproctis chrysorrhoea	n_1	O	3b	4	5	1	1	3	1	2	1	1	1	1	1	0
	Lymantria dispar	c	O	3b	4	5	1	1	2	1	2	1	1	1	1	1	0
Saturniidae	*Actias selene*	c	C	3a	5	6	1	1	3	0	1	1	1	0	1	0	0
	Antheraea mylitta	c	C	3a	5	7	1	1	3	0	1	1	1	0	1	0	0
	Antheraea pernyi	c	C	3a	4	5	1	1	2	1	1	1	2	0	1	1	0
	Antheraea roylei	c	C	3a	4	6	1	1	3	1	1	1	1	0	1	0	0
	Antheraea yamamai	c	C	3a	5	9	0	0	3	0	1	1	1	0	1	0	0
	Attacus atlas	c	D	3a	4	5	1	1	2	1	1	1	1	0	1	0	0
	Automeris viridescens	c	D	3b	4	6	1	0	4	0	1	1	1	0	0	0	0
	Caligula eucalyptae	c	D	3b	6	7	0	1	3	0	1	1	1	0	0	0	0
	Caligula grotei	c	D	3b	5	6	1	2	4	0	1	1	1	0	1	0	0
	Callosamia promethea	c	D	3a	6	8	1	0	2	0	1	1	2	0	1	0	0
	Cricula andrei	c	C	3b	6	7	0	0	5	0	1	1	0	0	0	0	0
	Dictyoploca japonica	c	D	3b	5	6	1	1	3	0	1	1	1	0	1	0	0
	Dictyoploca simla	c	D	3b	3	5	1	1	3	1	2	1	1	0	1	1	0
	Graellsia isabellae	c	C	3b	4	6	1	1	3	0	1	1	1	0	1	0	0
	Loepa katinka	c	D	3a	5	7	0	0	4	0	1	1	1	0	0	0	0
	Philosamia cynthia	c	C	3a	5	7	1	1	2	0	1	1	1	0	1	1	0
	Rhodinia fugax	c	C	3b	5	6	1	1	3	0	1	1	1	0	1	0	0
	Rothschildia forbesi	c	D	3a	5	6	1	1	2	0	1	1	1	0	1	1	0
	Rothschildia jacobeae	c	C	3a	5	7	1	0	1	0	1	1	1	1	1	0	0
	Rothschildia orizaba	c	C	3a	6	7	1	1	1	0	1	1	1	0	1	0	0
	Samia cecropia	c	C	3a	6	7	0	0	2	0	1	1	1	0	1	0	0
	Samia rubra	c	D	3a	6	7	0	0	3	0	1	1	1	0	1	0	0
	Saturnia pavonia	c	C	3a	3	5	2	3	1	1	1	1	2	0	1	1	0
	Telea polyphemus	c	C	3a	7	8	0	0	2	0	1	1	1	0	1	0	0
	Tropea luna	c	D	3b	4	6	1	1	4	0	1	1	1	0	1	0	0
Bombycidae	*Bombyx huttoni*	c	A	1	6	6	1	0	3	1	1	1	1	0	1	1	0
	Bombyx mandarina	c	A	1	5	5	1	1	3	1	1	1	2	0	1	1	0
	Bombyx meridionalis	c	A	1	6	4	1	1	3	1	1	1	1	0	1	1	1
	Bombyx mori	c	A	1	6	6	1	1	3	1	1	1	1	0	1	0	0
	Rondotia menciana	c	A	3b	5	5	1	1	3	1	1	1	1	0	1	0	1
Thaumetopoeidae	*Anaphe infracta*	c_1	E	2a	6	8	1	1	1	0	1	1	1	0	1	0	0
	Anaphe moloneyi	c_1	E	2a	8	9	1	0	0	0	1	1	1	0	0	0	0
	Anaphe reticulata	c_1	E	2a	5	7	1	0	1	1	0	1	1	1	1	1	0
	Anaphe venata	c_1	D	2a	8	10	1	0	0	0	0	0	1	0	0	0	0
	Hypsoides unicolor	c_1	A + D	2a	7	8	0	0	2	1	1	1	1	0	0	0	0
	Thaumetopoea pityocampa	n_1	F	4	4	3	1	2	4	1	1	1	1	1	2	1	0
Lacosomidae	*Cicinnus despecta*	c	D + H	n.d.	5	5	1	1	4	1	1	2	1	0	1	0	0
Nymphalidae	*Nymphalis io*	n_1	O	3b	4	5	1	1	1	0	1	1	1	0	1	1	0

TABLE 1 (*contd.*)

Family	Species	Form of fibroin	Method of Preparation	X-ray Group	Gly	Ala	Val	Leu/Ileu/Phe	Ser	Thr	Asp	Glu	Tyr	Pro	Arg	Lys	His
				CLASS: INSECTA													
Order: Lepidoptera (*contd.*)																	
Papilionidae	*Papilio machaon*	p	A	3b	5	8	1	1	2	1	1	0	1	0	1	1	0
Galleriidae	*Achroia grisella*	c	A	3b	4	5	1	2	3	0	1	1	0	1	1	1	0
	Galleria mellonella	c	A	3b	5	5	1	2	4	1	1	1	0	1	1	0	0
Pyraustidae	*Pyrausta sp.*	c	A	n.d.	4	7	1	1	2	1	1	2	0	1	0	0	0
Lasiocampidae	*Braura truncata*	c	A or H	3a	6	4	0	1	3	1	2	1	1	0	1	1	1
	Catalebeda violescens	c	C	3a	5	5	1	0	3	1	2	1	1	0	1	1	1
	Dendrolimus pini	c	A	3b	5	5	1	0	3	1	2	1	1	0	1	1	0
	Eriogaster lanestris	n_1	A	3b	4	5	1	1	3	1	1	1	1	0	1	1	0
	Eriogaster lanestris	c	A	3b	4	5	1	1	3	1	1	1	1	0	1	1	0
	Gastropacha quercifolia	c	A	3b	5	5	1	1	1	0	2	0	1	0	2	1	1
	Grammodora nigrolineata	c	C	3b	4	5	1	1	2	1	1	2	2	0	2	1	0
	Lasiocampa quercus	c	F + H	4	4	5	1	1	3	1	1	2	1	0	1	0	0
	Malacosoma neustria	c	A	3b	4	4	1	1	3	1	1	1	1	0	1	1	0
	Pachymeta flavia	c	H	3b	5	5	0	0	4	1	2	1	1	0	2	1	0
	Pachypasa otus	c	A	3b	5	5	1	1	2	1	2	1	1	0	2	1	0
	Philudoria potatoria	c	A	3b	5	4	1	1	3	1	1	1	1	0	1	1	1
	Trabala vishnu	c	C	3b	4	5	1	1	3	1	1	1	1	0	1	1	0
Psychidae	*Clania sp.*	c	A + D	2b	4	7	1	1	2	1	1	1	1	1	1	1	1
Cosmopterigidae	*Mompha ochracella*	c	A	3b	5	5	1	1	3	1	1	2	0	0	1	1	0
Yponomeutidae	*Yponomeuta evonymella*	c	A	3b	4	5	1	1	4	1	1	1	1	0	1	1	0
Gracillariidae	*Lithocolletis sp.*	f	A	n.d.	6	5	0	0	2	0	1	3	1	0	1	0	0
Plutellidae	*Argyresthia sp.*	c	A	3b	4	4	2	3	4	1	1	1	1	1	1	0	0
Lyonetiidae	*Leucoptera spartifoliella*	c	B	n.d.	6	6	1	0	4	1	1	1	1	0	0	0	0
Order: Hymenoptera																	
Braconidae	*Apanteles bignelli*	c	O	4	2	4	1	1	4	1	5	1	1	0	1	1	1
	Apanteles spurius	c	O	n.d.	2	3	1	1	4	1	3	1	1	0	1	1	1
				CLASS: ARACHNIDA													
Order: Araneae																	
Argiopidae	*Nephila madagascariensis*	g	A	3b	6	4	1	1	1	0	1	2	1	0	2	1	1
	Nephila senegalensis	c_2	G	5	2	5	1	2	4	1	1	2	1	0	1	1	1
Theraphosidae	*Avicularia avicularia*	n_2	G	4	3	6	1	2	3	1	1	2	1	1	1	1	0
	Scodra griseipes	n_2	G	4	4	6	1	1	4	1	1	1	1	0	1	1	0
	Tapinauchenius plumipes	n_2	G	4	4	6	1	1	4	1	1	1	1	0	1	1	0
Eusparassidae	*Torania variata*	c_2	G	n.d.	2	5	1	1	6	1	1	2	1	0	1	0	0

* stretched to β-form. n.d. = not determined.

Key to form of fibroin: c = Normal cocoon; c_1 = filaments from species producing a communal cocoon encasing individual cocoons; c_2 = egg cocoon; e = egg-stalk; f = *Lithocolletis* filaments, which are spun to enclose pupating larvae within a leaf shelter; g = supplied reeled, functional form unknown; n_1 = nest fibroins from species building communal nests during larval period; n_2 = spider nests; p = *P. machaon*, which supports itself by a filament during pupation.

same genus. Sometimes, as in *Thaumetopoea pityocampa*, the larvae produce a filamentous nest-fibroin, but make a cocoon which is paper-like in structure and appears to contain no filaments. In other species, for example in *Lasiocampa quercus*, the cocoon consists of a scaffolding of filaments cemented and filled-in with a second protein material and with

inorganic material, both of which come from the Malpighian glands and are non-fibrous in form. The fibroin of *Papilio machaon* is the silken girdle with which the pupa is attached to a reed-stalk.

The Arachnida produce various fibroins which may be in the form of a loose cocoon surrounding the group of eggs, or a snare or web, or a nest in which the spider rests. The form of these used in our analyses is shown in Table 1.

(b) *Purification of materials*

The need to purify the samples comes from the presence in most of them of a second protein, sericin, on the surface of the filaments, and from the frequent contamination of the silk with leaves and other debris. Sericins are normally soluble in hot, dilute alkaline solutions, but some silks require much more vigorous treatment than others to free them from sericin. The varying resistance of the different fibroins to chemical attack by alkali also makes it necessary to adapt the treatments to meet the needs of each fibroin and, with those fibroins that are susceptible to alkaline attack, the enzyme papain or a neutral or acid detergent was used. The key to the treatments is as follows:

A. Boiling soap solution ($\frac{1}{2}$ to 1%) for $\frac{1}{2}$ to 1 hr.

B. Boiling solution of soap (1%) and sodium bicarbonate (1%) for $\frac{1}{4}$ to $\frac{1}{2}$ hr.

C. Boiling solution of soap ($\frac{1}{2}$%) and sodium carbonate (1%), followed by soap ($\frac{1}{2}$%), for a total of 1 to 2 hr.

D. Boiling solution of soap ($\frac{1}{2}$ to 1%) and sodium carbonate (0·3 to 1%) for 2 hr.

E. Boiling solution of sodium carbonate (5%) for $1\frac{1}{2}$ hr followed by boiling soap (1%) and sodium carbonate ($\frac{1}{2}$%) for 1 hr.

F. Hot (80°C) aqueous Lissapol C† (1%) at a slightly acid reaction for $\frac{1}{4}$ to $\frac{1}{2}$ hr.

G. Warm dilute neutral solution of Teepol X‡ (1%), for $\frac{1}{4}$ to $\frac{1}{2}$ hr.

H. Papain buffered at pH 7 to 8, activated with ascorbate (40°C) for 4 to 24 hr.

O. Not purified, generally because only a very small sample was available.

With some of the fibroins, examination of the products under a low-power microscope showed the presence of insect hairs and, with some species, fragments of resistant material from the Malpighian glands; hand-picking with fine forceps was then necessary to obtain a pure sample of the fibroin.

It may be pointed out that the selection of the conditions for degumming a new fibroin is highly empirical. The usual procedure is to try the milder conditions first and, if these fail, to resort to more drastic methods. Consideration of the behaviour of related species is sometimes helpful in the choice of conditions; treatment is continued for as long as is necessary for the individual filaments to be thoroughly separated from each other and then for a further similar period. After appropriate treatment, the fibroins were thoroughly washed in several changes of hot water and then equilibrated with very dilute acetic acid and washed. Finally they were extracted with methanol or ethanol and with ether to remove traces of colouring matter or fats.

(c) *Analytical methods*

Quantitative amino acid analyses have been made either by the modified dinitrophenyl (DNP) method developed in these laboratories, or by the methods of ion-exchange chromatography developed by Moore & Stein. A preliminary account of the DNP method has already been given (Lucas *et al.*, 1955, 1957*b*) and a detailed account, with supporting analytical data, is in course of preparation. The general technique for ion-exchange chromatography closely followed that of Spackman, Moore & Stein (1958) except that the resin used was Zeo-Karb 225, 8% cross-linked. Estimation of the amino acids following their separation by this column was made by the ninhydrin—hydrindantin method described by Moore & Stein (1954).

Analyses for arginine and tyrosine were confirmed by the colorimetric methods of Rosenberg, Ennor & Morrison (1956) and of Ceriotti & Spandrio (1957). Tryptophan was estimated spectrophotometrically by the method of Goodwin & Morton (1946).

† Imperial Chemical Industries. ‡ Shell Chemicals.

The semi-quantitative determinations of the amino acids by paper chromatography were made on the total hydrolysates of the fibroins obtained by boiling them for 24 hr in 6 N-hydrochloric acid. Whatman No. 1 paper was used, with a mixture of butanol—acetic acid—water as the moving phase in one direction, and phenol—water in the other. 8-Hydroxyquinoline, or potassium cyanide, was used as antioxidant with the phenol-water system. Ammonia was sometimes included to speed up the movement of the basic amino acids.

After the papers had been sprayed with ninhydrin, the relative intensities of the colours of the various spots were estimated by visual judgement. Errors involved in this method are due not only to the subjective assessment of the observer but also to the differential colour yields of the amino acids. In particular we have found that glycine has a lower colour yield on paper chromatograms than most of the other amino acids. The visual estimate of the colour intensity of glycine was therefore corrected to allow for this. In reporting the results the relative intensities are given on a numerical basis in such a way that the total is approximately 20.

In addition to the limitations mentioned above a further restriction on accuracy is imposed by the wide range of concentrations of amino acids present in most fibroins, with glycine, alanine and serine always predominating. Some of the minor constituents are present in only trace amounts, but these have been included and accorded the lowest value of 1. It will therefore be understood that this value of 1 embraces a relatively wider range of concentrations in a proportionate sense than the numbers of the rest of the series.

3. Results

The results obtained from the quantitative estimation of the amino acid composition of the various fibroins are shown in Table 2, the various species being grouped according to their biological classification. Warwicker's X-ray grouping is also shown in the table. All results are expressed as grams of amino acid nitrogen per 100 grams of fibroin nitrogen. In certain instances the amount of material available has been very small, and it has not then been possible to obtain complete analytical data. The results for serine and threonine have been corrected for decomposition during hydrolysis by assuming that this occurs at the rates found by Rees (1946).

The amount of ammonia in the hydrolysates was determined in those analyses carried out on resin columns, but is not given by the DNP method. Where values for ammonia are given they have been reduced by the same amount as the serine and threonine values have been increased, since decomposition of these hydroxy amino acids leads to an equivalent amount of ammonia. The remaining ammonia probably arises mainly from decomposition of amide groups, though a small amount may arise from decomposition of other amino acids and from external sources. In many fibroins the amount of material available for analysis was too small to enable direct determination of amide nitrogen to be made, but an indication of the amounts of acid amide present in some of the fibroins can be obtained from the ammonia values, since most of the ammonia comes from hydrolysis of asparagine and glutamine.

Most of the fibroins whose amino acid compositions were determined quantitatively were also investigated by paper chromatography, and these results are given in Table 1. A comparison of the two sets of results indicates the accuracy that can be attributed to the semi-quantitative assessments from the paper chromatography. The correlation is, in general, satisfactory though the ratio of glycine to alanine of the *Bombyx* genera, which should be greater than 1, is assessed by paper chromatography as about equal to 1, possibly because the correction applied to compensate for the low colour intensity on paper of ninhydrin-treated glycine, as judged by the eye, is too low when the concentration of glycine relative to the other amino acids is as high as it is with the Bombycidae.

TABLE 2

Quantitative analyses of various fibroins

Results expressed as grams of amino acid nitrogen per 100 grams protein nitrogen

	Chrysopidae	Arctiidae	Agrotidae	Galleriidae	Saturniidae									Bombycidae		Thaumetopoeidae				Lasiocampidae				Psychidae	Argiopidae	
	*Chrysopa flava**	*Arctia caja*	*Bena prasinana*	*Galleria mellonella*	*Antheraea pernyi*	*Antheraea mylitta*	*Antheraea yamamai*	*Philosamia cynthia*	*Dictyoploca japonica*	*Cricula andrei*	*Loepa katinka*	*Callosamia promethea*	*Attacus atlas*	*Bombyx mori*	*Bombyx meridionalis*	*Anaphe moloneyi*	*Anaphe venata*	*Anaphe infracta*	*Thaumetopoea pityocampa*	*Lasiocampa quercus*	*Pachypasa otus*	*Pachymeda flavia*	*Braura truncata*	*Clania sp.*	*Nephila madagascariensis*	*Nephila senegalensis*
X-ray group: Method:	†3a D	4 D/M	1 M	3b M	3a §	3a D	3a M	3a M	3b D/M	3b D	3a D/M	3a D	3a M	1 ‡	1 D	2a D	2a D	2a D/M	4 D	4 D/M	3b D	3b D	3a D	2b M	2b D	5 M
Glycine	23·5	16·6	40·3	29·2	23·7	23·5	23·4	27·7	20·3	25·0	23·5	28·8	28·6	43·7	44·4	41·7	40·1	28·3	18·8	14·0	27·1	26·3	27·5	33·2	35·3	10·5
Alanine	20·2	17·4	23·4	22·9	39·4	36·9	40·0	42·2	34·7	31·0	36·7	39·8	41·8	28·8	29·5	52·1	51·6	53·2	10·9	16·8	21·1	28·6	23·2	30·4	27·9	25·3
Valine	n.d.	3·0	1·9	4·8	0·6	0·8	0·7	0·5	2·1	0·9	0·7	n.d.	0·9	2·2	2·1	1·0	0·3	0·6	2·3	4·0	0·6	1·1	0·4	1·5	0·8	2·0
Leucine	n.d.	1·9	1·2	6·2	} 0·7	} 0·7	0·2	0·2	} 3·9	} 1·6	2·0	n.d.	0·4	0·5	} 1·5	} 0·3	} 0·2	} 0·5	3·4	2·2	} 1·7	1·6	} 1·6	0·8	} 2·5	5·7
*Iso*leucine	n.d.	1·7	0·8	3·2			0·3	0·3			0·6	n.d.	0·3	0·7					3·1	1·4		0·9		1·2		2·2
Serine	40·7	8·6	12·9	15·3	10·5	9·8	8·6	4·5	12·2	14·0	8·1	6·5	6·8	11·9	11·3	0·8	0·7	2·1	17·3	12·8	7·2	8·8	6·8	9·3	3·7	19·9
Threonine	3·0	2·8	1·4	1·7	0·1	0·9	0·6	0·5	1·1	0·6	0·4	0·5	0·7	0·9	1·0	0·6	0·4	0·6	2·3	2·4	0·8	1·0	0·8	1·5	0·5	3·8
Aspartic acid	5·7	8·0	2·0	1·9	4·2	5·7	4·4	3·1	4·3	} 8·5	4·9	} 5·3	3·7	1·3	1·5	} 0·7	} 1·2	} 3·0	9·2	4·4	8·8	6·1	7·8	2·6	0·8	2·8
Glutamic acid	0·8	4·4	2·9	0·5	0·7	0·9	0·6	0·7	1·9		1·3		0·6	1·0	1·1				3·8	5·0	0·9	1·3	0·9	2·4	10·1	8·6
Arginine	1·8	12·6	4·1	4·8	9·2	13·3	10·2	6·6	7·7	9·8	5·7	n.d.	5·5	1·8	1·9	2·1	1·9	3·8	10·0	14·6	25·5	11·3	25·3	4·1	8·5	6·7
Histidine	n.d.	3·2	0·4	0·1	2·2	n.d.	2·5	3·8	5·4	n.d.	nil	n.d.	4·6	0·5	n.d.	n.d.	n.d.	1·4	0·1	4·5	n.d.	0·8	n.d	0·7	n.d.	0·2
Lysine	n.d.	3·9	0·7	0·4	0·2	n.d.	0·8	0·6	1·2	0·3	3·6	n.d.	1·3	0·6	0·4	n.d.	n.d.	0·4	0·6	3·6	0·4	2·6	0·7	3·4	n.d.	1·0
Tyrosine	0·8	2·8	5·6	0·3	4·4	4·8	4·1	4·9	3·8	3·4	7·3	5·0	3·7	5·1	4·8	0·2	0·2	1·6	3·4	4·0	2·3	3·6	2·9	2·9	2·8	1·8
Phenylalanine	n.d.	1·0	0·3	0·2	0·5	0·3	0·2	0·1	0·4	3·2	nil	n.d.	nil	0·6	1·0	0·2	0·3	0·2	1·1	0·8	0·4	n.d.	0·7	0·2	0·5	3·3
Proline	n.d.	2·5	0·3	4·3	0·3	n.d.	0·1	0·3	0·6	n.d.	nil	n.d.	0·3	0·3	n.d.	n.d.	n.d.	0·9	n.d.	n.d.	nil	n.d.	n.d.	0·9	n.d.	n.d.
Tryptophan	0·5	n.d.	n.d.	n.d.	2·0	3·0	n.d.	n.d.	1·8	n.d.	n.d.	n.d.	n.d.	0·3	n.d.	0·2	0·4	2·1	0·7	n.d.	0·3	n.d.	n.d.	n.d.	n.d.	n.d.
Amide N	n.d.	n.d.	n.d.	n.d.	n.d.	1·5	n.d.	n.d.	1·7	n.d.	n.d.	n.d.	n.d.	nil	n.d.	n.d.	n.d.	n.d.	9·2	5·7	2·1	2·9	n.d.	n.d.	n.d.	n.d.
Ammonia	n.d.	9·3	4·4	3·3	0·7	n.d.	0·8	1·6	n.d.	n.d.	2·1	n.d.	1·5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5·3	n.d.	n.d.
TOTAL	97·0	99·7	102·6	99·1	99·4	102·1	97·5	97·6	103·1	98·3	96·8	85·9	100·7	100·2	100·5	99·9	97·3	98·7	96·2	96·2	99·2	96·9	98·6	100·4	93·4	93·8
Total N %	16·7	13·6	17·0	16·1	18·9	18·6	18·9	18·4	17·7	n.d.	17·0	19·1	18·9	18·3	n.d.	20·4	20·2	18·4	15·1	17·6	19·5	16·9	n.d.	16·8	17·5	16·1

† = when stretched to β-form. n.d. = not determined. § Schroeder & Kay (1955). * Lucas, Shaw & Smith (1957*a*). ‡ Lucas, Shaw & Smith (1958). Analytical methods: D = DNP method, M = Moore & Stein method.

The larvae of *Euproctis chrysorrhoea* (Lymantriidae) and *Eriogaster lanestris* (Lasiocampidae) produce fibroin in the form of a nest, as well as cocoon fibroin. Examination of these two forms by paper chromatography showed them to be closely similar in amino acid composition (Table 1).

4. Discussion

In recent years there has been an increasing interest in what has been called "protein taxonomy". Crick (1958) has suggested that the amino acid sequences of a particular protein may be "the most delicate expression possible of the phenotype of the organism, and that vast amounts of evolutionary information may be hidden away within them." Such a contention can be considered in the light of the work of Sanger and co-workers on various mammalian insulins (Sanger & Tuppy, 1951; Brown, Sanger & Kitai, 1955), and of Li and his group (Li, 1956, 1957) on the structure of various anterior pituitary hormones. Anfinsen (1959) gives additional examples and discusses their evolutionary significance.

In only a very few proteins, however, has the actual sequence of the amino acids been determined, and it is usually necessary to rely on some secondary characteristic, such as amino acid composition, or, in mixed proteins, the proportions of the individuals composing them, before attempting a classification and comparing it with conventional classifications based on morphological characters. Sibley (1960), from studies of the electrophoretic mobility of the various proteins present in the egg-white of a large number of species of the class Aves, has suggested that the molecular structure of proteins is phylogenetically conservative, and may be a valuable indication of relations existing between groups of birds. His results, however, contain a number of examples where the egg-whites of closely related species (for example various gannets, Sulidae) have widely differing electrophoretic patterns.

Consideration of the quantitative amino acid analyses of Table 2, and of the semi-quantitative data of the chromatograms given in Table 1, shows that no precise relations exist between the amino acid compositions of the various fibroins and their biological and X-ray classifications. However, certain broadly based conclusions can be drawn. One common factor running through all the results is that the three simplest natural amino acids, glycine, alanine, and serine, constitute a high proportion of each fibroin. For the fibroins so far investigated by us, the sum of these amino acids is never less than 42·6% (*Arctia caja*) and may be as high as 94·6% (*Anaphe moloneyi*).

The Saturniidae form a group in which the alanine content is always greater than the glycine content, the ratio of alanine to glycine ranging from 1·71 for *Antheraea yamamai* and *Dictyoploca japonica* to 1·24 for *Cricula andrei*. All the members of the Saturniidae have contents of glycine *plus* alanine *plus* serine that are closely similar, and lie between 67% and 76% of the total amino acid content, and they all belong to the closely related X-ray groups 3a and 3b. However, fibroins belonging to other families, for example, *Pachypasa otus* and *Pachymeta flavia* (Lasiocampidae), *Galleria mellonella* (Galleriidae), and *Nephila madagascariensis* (Argiopidae) which belongs to a different class from the others, also give X-ray diffraction patterns that place them in group 3b, yet all have a higher content of glycine than of alanine. There is thus no general correlation between the X-ray grouping and the amino acid distribution of the fibroins and it is not yet evident what factors dictate the composition of the ordered, crystalline, parts of any fibroin.

There are two examples in which fibroins produced by moths that are widely separated taxonomically have similar amino acid compositions and are also placed

in the same groups by X-ray examination. The first is remarkable and relates to the close similarity between the composition of the fibroin of commerce (*B. mori*) and that of *Bena prasinana* (the Green Silver Lines moth). The latter is a small British moth apparently unrelated to *B. mori*, yet the fibroin of its cocoon has an amino acid composition very similar to that of *B. mori* and an X-ray diffraction pattern that reveals a structure closely similar to, but more highly oriented than, that of *B. mori*. Previously, only a few nearly-related members of the Bombycidae have shown close similarity in amino acid distribution and X-ray diffraction pattern to the fibroin typified by *Bombyx mori*, and the discovery that the fibroin of *Bena prasinana* belongs to that group is therefore of considerable interest.

Secondly, the species *Lasiocampa quercus*, *Thaumetopoea pityocampa* and *Arctia caja* belong to three different families, yet the amino acid analyses of their fibroins are similar, particularly with respect to their glycine and alanine contents, which are relatively low compared with other fibroins, and they are placed by X-rays in the same group 4.

The Anaphe group of fibroins, represented mainly by African species that spin a large communal cocoon that may contain several hundred small cocoons of the individual larvae, is remarkable in that glycine and alanine together constitute over 90% of the total protein in the species *Anaphe moloneyi* and *Anaphe venata*, and over 80% in the species *Anaphe infracta*. In all these species over half the protein is composed of alanine. Another related genus, *Hypsoides*, also makes a large communal cocoon and its fibroin, too, is composed mainly of glycine and alanine. All these fibroins are placed by X-rays in the same group 2a. However the Psychida *Clania sp.*, which is placed in the closely related Group 2b by X-rays, has an amino acid distribution quite unlike that of the Anaphe fibroins.

The fibroins of the two spiders *Nephila madagascariensis* and *Nephila senegalensis*, which are taxonomically closely related, differ in amino acid composition and X-ray classification. This may be a reflection of the different functional form of the two fibroins and of a difference in the type of gland from which they are produced.

The results of Kirimura (1957) and those of Duchâteau *et al.* (1960) may be compared with ours when the same fibroins are involved. These workers employed microbiological assay methods for their amino acid analyses. However, the analyses of Duchâteau *et al.*, for the fibroins of *Loepa katinka*, *Cricula andrei*, *Philosamia cynthia*, and *Attacus atlas*, do not agree with ours, and in particular their values for alanine are comparatively very low. Hence their attempts at a "molecular taxonomy," which was based primarily on the total of alanine *plus* glycine in the fibroins, must be considered as suspect. The results of Kirimura, on the other hand, support ours, and in particular his values for *Philosamia cynthia* (syn. *Attacus ricini*) and *Antheraea yamamai* are in excellent agreement with ours.

It seems fairly certain that, whilst the fibroins produced by closely related species belonging to the same genera may have closely similar amino acid compositions (for example members of the *Bombyx*, *Antheraea*, and *Anaphe* genera), this is not an exclusive characteristic, because fibroins from other genera or from other families may also closely resemble them in amino acid composition, or be placed in the same group by X-ray analysis. Such classification by X-ray diffraction patterns is limited because, as shown by Drucker, Hainsworth & Smith (1953) for *Bombyx mori* fibroin, the amino acid composition of the ordered, "crystalline," regions of a fibroin may not be related to the total composition in any proportionate sense, and may indeed contain only a few of the range of amino acids present in the fibroin.

A feature of the analyses presented here is the variation in amino acid composition shown by the cocoon fibroins of insects. Anfinsen (1959) has suggested that the permissible degree of variation in proteins such as the collagens, elastins, hair, and skin is likely to be larger than that permissible in the "dynamic" proteins that are closely associated with the immediate tasks of keeping the organism alive. The work of Eastoe & Leach (1958) and of Watson (1958) on the composition of vertebrate and invertebrate collagens of widely differing origins shows this to be true, for although certain important features are common to all, such as the high contents of proline *plus* hydroxyproline, there are distinct variations in the distribution of other amino acids, particularly amongst the minor constituents. Divergencies in composition are also apparent amongst keratins of different species (Ward & Lundgren, 1954), the cystine content of keratins being particularly variable.

The fibroins that provide the structural basis of the cocoons of different species of pupating insects perform essentially the same function for each. This function is the production of filaments from which a rigid structure can be made in which the pupa is protected from predators, extremes of temperature, physical shock, microbiological attack, and the general rigours of climate. It is remarkable that the wide variation in amino acid composition that has been found is perfectly compatible with this function. As far as we know, no other comparable group of proteins exhibits such variations. It is, perhaps, to the type of environment in which the insect pupates and to its attendant hazards that attention should be directed in seeking an explanation of the diversification of fibroin as an evolutionary process.

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Comparative Studies of Fibroins

II. The Crystal Structures of Various Fibroins

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An X-ray examination of the fibres produced by the silk-spinning organisms belonging to the orders Lepidoptera and Araneae has shown that there are at least five main types of fibroins recognizable by their X-ray diagrams.

It is shown that probably all the fibroins are made up of anti-parallel pleated sheets of polypeptide chains, the difference between the fibroins being attributed to the variation in, and sequence of, the amino-acid residues composing the polypeptide chains. Possible structures for the crystalline regions of the fibroins are postulated on consideration of the composition, and possible modes of packing of the anti-parallel pleated sheets.

I. Introduction

In a previous X-ray investigation of fibroins (Warwicker, 1956) produced by the larvae of moths of nine different species it was concluded that the fibroins could be divided into three groups on the basis of their X-ray diagrams, and this classification was found to agree with the biological classification of the moths as given by Bock & Pigorini (1938). More recently, however, Lucas, Shaw & Smith (1960) have carried out chemical work on a large range of silk-like filaments, and as a result have extended the definition of fibroin (Lucas, Shaw & Smith, 1958, 1960) to include filaments spun by species belonging to the classes Insecta and Arachnida—in particular to filaments from species of the orders Lepidoptera (moths) and Araneae (spiders). In the present work a much larger range of fibroins was available than previously and it has been discovered that five main groups of fibroins can be distinguished by their X-ray diagrams. Although there is some correspondence between the X-ray and biological classifications it is now evident that the correspondence is not complete and that anomalies occur. Suitable unit cells can be chosen by which the characteristic X-ray diagrams can be indexed, and if these results, together with other X-ray and chemical evidence, are taken into account, tentative structures for the crystalline regions of the fibroins can be proposed.

2. Experimental

The fibroins used in this work were cleaned and, wherever possible, degummed (Lucas, Shaw & Smith, 1960) before X-ray photographs were taken. Standard 25 mg bundles of fibres one inch long were made if possible, but some samples were too small for this purpose and then small bundles of parallel fibres were made, often gummed together with a dilute solution of gum tragacanth.

X-ray photographs of the standard bundles were taken with the bundle held taut in a special frame; the small gummed bundles were mounted vertically on the goniometer of the X-ray camera and rotated during exposure. Further X-ray photographs were taken for all the typical fibroins with the fibre held horizontally and at an angle to the X-ray

beam such that the sixth-order reflexion of the fundamental fibre repeat was recorded. A cylindrical camera of radius 3 cm and nickel-filtered copper Kα radiation were used throughout.

The diffractometer curves were obtained with a proportional counter and a counting chain with a resolving time of 5 μsec. The maximum counting rate did not exceed 50 counts/sec and therefore the correction for resolving time was negligible. The statistical error was between 1 and 1·5%.

3. Classification of the Fibroins

On examination of the X-ray photographs it was found that by a comparison of the spacings, especially of those corresponding to the two principal equatorial arcs, the fibroins could be classified into five groups. However, in two of these groups it was found that, although the spacings corresponding to these principal arcs were the same, there were fibroins whose X-ray diagrams differed in the relative intensity of the main arcs. Since the orientations of the arcs of the typical fibroins were found to be similar, this difference in relative intensity suggested a fundamental structural difference, and in consequence these groups of fibroins were divided into two groups on the basis of the relative intensity of the two main equatorial diffraction spots. Thus groups 2a and 3a include those fibroins whose X-ray diagrams show the two main equatorial diffraction spots to be equally intense, and groups 2b and 3b include those fibroins, the X-ray diagrams of which show a difference in intensity for these spots

Table 1

Family or sub-family	Species
	Group 1
Bombycidae	*Bombyx huttoni*
	Bombyx mandarina
	Bombyx meridionalis
	Bombyx mori
Cymbidae	*Bena prasinana*
	Group 2a
Thaumetopoeidae	*Anaphe infracta*
	Anaphe moloneyi
	Anaphe reticulata
	Anaphe venata
	Hypsoides unicolor
	Group 2b
Psychidae	*Clania sp.*
	Group 3a
Lasiocampidae	*Braura truncata*
	Catalebeda violescens
Saturniidae	*Actias selene*
	Antheraea mylitta
	Antheraea pernyi
	Antheraea roylei
	Antheraea yamamai
	Attacus atlas
	Callosamia promethea
	Loepa katinka
	Philosamia cynthia
	Rothschildia forbesi

TABLE 1 (*contd.*)

Family or sub-family	Species
	Group 3a
	Rothschildia jacobeae
	Rothschildia orizaba
	Samia cecropia
	Samia rubra
	Saturnia pavonia
	Tela polyphemus
	Group 3b
Argiopidae	*Nephila madagascariensis*
Bombycidae	*Rondotia menciana*
Cosmopterigidae	*Mompha ochracella*
Galleriidae	*Achroia grisella*
	Galleria mellonella
Lasiocampidae	*Eriogaster lanestris*
	Dendrolimus pini
	Gastropacha quercifolia
	Grammodora nigrolineata
	Malacosoma neustria
	Pachymeta flavia
	Pachypasa otus
	Philudoria potatoria
	Trabala vishnu
Lymantriidae	*Euproctis chrysorrhoea*
	Lymantria dispar
Papilionidae	*Papilio machaon*
Plutellidae	*Argyresthia sp.*
Saturniidae	*Automeris viridescens*
	Caligula eucalypti
	Caligula grotei
	Cricula andrei
	Dictyoploca japonica
	Dictyoploca simla
	Graellsia isabellae
	Tropaea luna
	Rhodinia fugax
Yponomeutidae	*Yponomeuta evonymella*
	Group 4
Arctiidae	*Arctia caja*
Braconidae	*Apanteles bignelli*
Lasiocampidae	*Lasiocampa quercus*
Thaumetopoeidae	*Thaumetopoea pityocampa*
Theraphosidae	*Avicularia avicularia*
	Tapinauchenius plumipes (nest web)
	Scodra griseipes (nest web)
	Group 5
Argiopidae	*Araneus diadematus*
	Nephila senegalensis (cocoon)
	Species of Indian Spider
	Miscellaneous
Argiopidae	Species of spider
Eusparassidae	*Torania variata*
Nymphalidae	*Nymphalis io* (Group 3b?)

although not a difference in the corresponding spacing. It is not claimed that the fibroins in group 3b are identical, since other minor differences in the X-ray diagrams can be found, but the X-ray diagrams have the same general features. If the previous results of Warwicker (1956) or Trogus & Hess (1933) had been analysed in a similar way, three main groups and one sub-group of fibroins could have been distinguished, so that the present work reports two new main groups of fibroin not previously observed, making five main groups of fibroins in all.

The group labelled "miscellaneous" does not imply another group of fibroins, but contains those fibroins that gave such poor X-ray diagrams that they could not be classified with certainty.

Typical X-ray diagrams of the five main groups and one subgroup of fibroins are given in Plate I, and Table 1 gives the classification of the fibroins according to their type of X-ray diagram.

It is evident from Table 1 that the biological and the X-ray classifications of the fibroins are not identical. However, some correspondence does still occur, especially in groups 1, 2, 3, and 5, and to a smaller extent in the remaining groups.

Although the X-ray classification implies that the fibroins within a given group are of the same basic structural type, it is not improbable that variations in details of this structure occur for the different fibroins of the group. It is still important, therefore, to treat each fibroin as unique, although some information about its structure can be obtained by reference to the general structure implied by the X-ray classification.

4. Structure of Fibroins

The interplanar spacings corresponding to the arcs on the X-ray diagrams of all the typical fibroins were analysed, and it was found that some spacings were common to all fibroins. A list of these common spacings is given in Table 2. It is interesting

TABLE 2

Common spacings of typical fibroins

Group	Typical fibroin	Observed spacings (in Å) of 0*kl* reflexions								
		040	060	041	002	022	032	013	023	033
1	*Bombyx mori*	2·38	1·56	2·30	3·53	2·83	2·31	2·27	2·10	1·82
2a	*Anaphe moloneyi*	2·38	1·58	2·28	3·49	2·86	2·28	2·26	2·06	1·84
2b	*Clania sp.*	2·30	1·57	2·29	3·53	2·79	—	2·28	2·05	—
3a	*Antheraea mylitta*	2·38	1·58	2·28	3·44	2·92	2·29	2·25	2·07	1·84
3b	*Dictyoploca japonica*	—	1·58	2·23	3·45	2·92	2·29	2·28	2·09	1·88
4	*Thaumetopoea pityocampa*	2·36	1·55	—	3·40	—	—	2·26	2·10	1·88
5	*Nephila senegalensis*	2·35	1·57	2·19	3·43	2·77	—	2·23	2·06	1·87
	Calculated spacing	2·36	1·57	2·23	3·48	2·80	2·31	2·25	2·08	1·87

to note that the arcs on the third layer line of the X-ray diagrams form a characteristic feature of these diagrams for all fibroins and arise from planes with common spacings.

An explanation of these common spacings has already been given (Warwicker, 1956) in terms of the current view (Marsh, Corey & Pauling, 1955*a*,*b*) of the structure

of fibroins. According to this view the crystalline portion of the fibroins consist of anti-parallel pleated sheets of polypeptide chains packed into orthorhombic unit cells. Since in such structures two dimensions remain constant, viz. the distance between chains in the direction of hydrogen bonds, and the fibre repeat distance, common spacings must arise for those planes for which the index referring to the third dimension of the unit cell remains zero. That common spacings are found for all the fibroins seems reasonable proof that the same basic structure occurs in all of them.

Additional support for this conclusion is provided by measurement of the fibre repeat distance for all the typical fibroins. This was readily found to be $6{\cdot}95 \pm 0{\cdot}05$ Å for all the typical fibroins, from a measurement of the sixth-order reflexion of the fundamental fibre repeat found on the photographs taken with a horizontal bundle of fibres. Although small variations of this value are found for different fibroins the variation is too small to invalidate the conclusion that the fibre repeat distance is substantially a constant for all fibroins.

Since nothing has been postulated for the internal symmetry, it is convenient to treat the unique axis, the fibre axis, of the orthorhombic cell as the *c*-axis. The axis in the direction of the hydrogen bonds of the anti-parallel pleated sheets has been taken as the *b*-axis, leaving the *a*-dimension of the unit cell, the direction of the side-chains, to be determined for each class of fibroin.

The third dimension of the unit cell can be derived from the X-ray diagram if it is assumed that the two main equatorial reflexions can be indexed as 200 and 120. Unit cells derived in this way have been found satisfactory as a basis for indexing the X-ray diagrams of four of the five main groups of fibroins, viz. groups 1, 2a, 2b, 3a, 3b, and 5. To derive a satisfactory unit cell to interpret the X-ray diagrams of the fibroins of group 4, however, the two main equatorial reflexions have to be indexed as 210 and 120. The results are summarized in Table 3. It should be noted that because

TABLE 3

Dimensions of unit cells of silk fibroins in Å

Group	Representative Fibroin	*a*	*b*	*c* (Fibre axis)
1	*Bombyx mori*	9·3	9·44	$6{\cdot}95 \pm {\cdot}05$
2a	*Anaphe moloneyi*	10·0	9·44	$6{\cdot}95 \pm {\cdot}05$
2b	*Clania sp.*			
3a	*Antheraea mylitta*	10·6	9·44	$6{\cdot}95 \pm {\cdot}05$
3b	*Dictyoploca japonica*			
4	*Thaumetopoea pityocampa*	15·0	9·44	$6{\cdot}95 \pm {\cdot}05$
5	*Nephila seneyalensis*	15·7	9·44	$6{\cdot}95 \pm {\cdot}05$

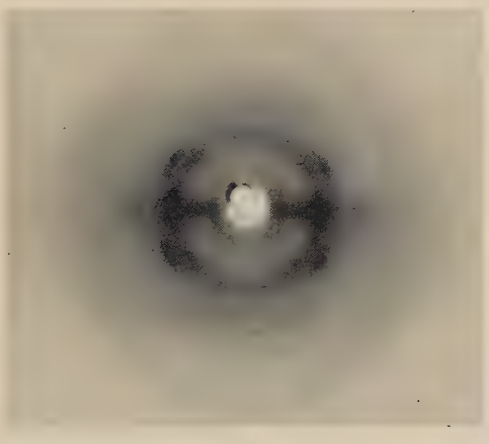
of the experimental difficulties the accuracy of the *a*-dimension is not high. Slightly greater accuracy was achieved for the typical members of groups 3a and 3b, because a clear 400 reflexion is given, from which a more accurate value of the *a*-dimension can be determined than from the 200 reflexion. However, the cells given are adequate for the indexing of the diagrams as can be seen by the results given in Tables 4 to 8 for a typical member of each of the groups 2a, 2b, 3b, 4, and 5. Results have not been included for groups 1 and 3a because they have been adequately covered by the

1

2

3

4



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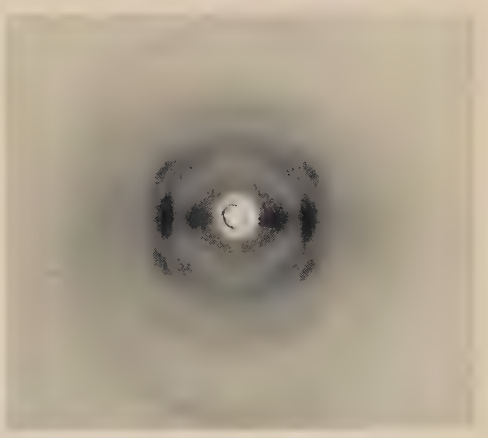

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PLATE I. Typical X-ray diagrams of fibroins

1. *Bombyx mori*
2. *Anaphe moloneyi*
3. *Antheraea mylitta*
4. *Dictyoploca japonica*
5. *Thaumetopoea pityocampa*
6. *Nephila senegalensis*

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TABLE 4

Data from the X-ray diagram of the fibroin from Anaphe moloneyi (*group 2a*)

Reflexion	θ	d	I	Index	d (calculated)
E_1	4·82°	9·16 Å	m	010	9·44 Å
E_2	8·93	4·96	vs	200	5·00
E_3	10·4	4·27	vs	120, 210	4·27, 4·42
E_4	18·88	2·38	w	040, 410	2·36, 2·42
E_5	21·51	2·10	vw	240	2·14
E_6	25·95	1·76	vw	250, 440	1·77, 1·72
E_7	29·17	1·58	w	060, 160, 620	1·57, 1·55, 1·57
I_0	6·26	7·06	w	001	6·96
I_1	10·9	4·07	wm	201	4·06
I_2	12·11	3·67	s	121, 211	3·64, 3·73
I_3	17·72	2·53	vw	321, 231	2·54, 2·49
I_4	19·74	2·28	w	041, 411	2·24, 2·28
I_5	21·51	2·10	w	421, 241	2·11, 2·04
II_0	12·75	3·49	m	002	3·47
II_1	15·62	2·86	ms	022, 212	2·80, 2·73
II_2	19·74	2·28	w	032, 132	2·33, 2·27
III_1	19·92	2·26	m	013	2·25
III_2	21·95	2·06	ms	023	2·08
III_3	24·74	1·84	vw	033	1·87

$a = 10{\cdot}0$ Å $b = 9{\cdot}44$ Å $c = 6{\cdot}95$ Å

TABLE 5

Data from the X-ray diagram of the fibroin from Clania sp. (*group 2b*)

Reflexion	θ	d	I	Index	d (calculated)
E_1	4·77°	9·26 Å	s	010	9·44 Å
E_2	8·82	5·02	s	200	5·00
E_3	10·40	4·27	vs	120, 210	4·27, 4·42
E_4	14·55	3·07	w	030, 310, 130	3·15, 3·00, 3·14
E_5	18·13	2·48	vw	400	2·50
E_6	19·56	2·30	vw	140, 040, 410, 330	2·30, 2·36, 2·42, 2·29
E_7	21·70	2·08	vw	240	2·14
E_8	29·34	1·57	vw	060, 160, 620	1·57, 1·55, 1·57
I_1	12·23	3·64	s	121, 211	3·64, 3·73
I_2	19·63	2·29	w	041, 411, 401	2·24, 2·28, 2·35
II_0	12·62	3·53	s	002	3·48
II_1	16·02	2·79	ms	212, 022, 202	2·73, 2·80, 2·85
III_1	19·79	2·28	w	013	2·25
III_2	22·10	2·05	ms	023, 123, 213	2·08, 2·04, 2·05

$a = 10{\cdot}0$ Å $b = 9{\cdot}44$ Å $c = 6{\cdot}95$ Å

TABLE 6

Data from the X-ray diagram of the fibroin from Dictyoploca japonica (*group 3b*)

Reflexion	θ	d	I	Index	d (calculated)
E_1	4·54°	9·73 Å	m	010	9·44 Å
E_2	8·27	5·35	s	200	5·30
E_3	10·20	4·35	vs	120, 210	4·32, 4·62
E_4	16·96	2·64	m	400	2·65
E_5	29·12	1·58	vw	060, 540	1·57, 1·62
I_1	10·58	4·20	ms	201	4·21
I_2	12·15	3·66	s	121, 211, 021	3·67, 3·85, 3·91
I_3	20·25	2·23	w	041, 331, 421	2·24, 2·23, 2·19
II_0	12·9	3·45	w	002	3·47
II_1	15·3	2·92	ms	022, 202	2·80, 2·91
II_2	19·67	2·29	vw	032, 132	2·33, 2·28
III_1	19·76	2·28	w	013	2·25
III_2	21·65	2·09	m	023	2·08
III_3	24·25	1·88	vw	033	1·87

$a = 10{\cdot}6$ Å $\quad b = 9{\cdot}44$ Å $\quad c = 6{\cdot}95$ Å

TABLE 7

Data from the X-ray diagram of the fibroin from Thaumetopoea pityocampa (*group 4*)

Reflexion	θ	d	I	Index	d (calculated)
E_1	4·67°	9·46 Å	s	010	9·45 Å
E_2	7·54	5·87	s	210	5·89
E_3	9·87	4·49	vvs	120, 310	4·51, 4·44
E_4	14·17	3·15	m	030, 130	3·15, 3·08
E_5	15·88	2·82	m	230	2·91
E_6	16·70	2·68	w	330	2·67
E_7	19·08	2·36	vw	040, 140, 430	2·36, 2·33, 2·42
E_8	27·19	1·69	w	900, 450	1·68, 1·69
E_9	29·72	1·55	wm	060, 160	1·57, 1·57
E_{10}	33·39	1·40	w	560, 750, 11,00	1·39, 1·42, 1·39
I_1	10·08	4·40	ms	211	4·49
I_2	11·99	3·71	s	121, 311	3·78, 3·75
I_3	20·93	2·16	m	141, 241, 621	2·22, 2·14, 2·11
II_0	13·09	3·40	ms	002	3·48
II_1	14·59	3·06	ms	212	2·99
II_2	18·37	2·44	w	322, 412	2·45, 2·47
II_3	20·00	2·25	w	232, 512, 422	2·23, 2·21, 2·25
III_0	19·90	2·26	w	013	2·25
III_1	21·53	2·10	m	023	2·08
III_2	24·12	1·88	w	033	1·87

$a = 15{\cdot}0$ Å $\quad b = 9{\cdot}44$ Å $\quad c = 6{\cdot}95$ Å

TABLE 8

Data from the X-ray diagram of the fibroin from Nephila senegalensis (*group 5*)

Reflexion	θ	d	I	Index	d (calculated)
E_1	5·73°	7·71 Å	s	200	7·85 Å
E_2	9·74	4·55	vs	120, 310, 020	4·52, 4·58, 4·72
E_3	16·0	2·80	m	230	2·92
E_4	19·1	2·35	w	040, 140	2·36
E_5	29·4	1·57	m	060, 160, 10,00	1·57, 1·57, 1·57
I_1	9·72	4·56	ms	211	4·56
I_2	11·92	3·73	s	021, 121, 311	3·91, 3·79, 3·82
I_3	20·62	2·19	w	041, 141, 621	2·23, 2·21, 2·17
II_0	12·97	3·43	w	002	3·47
II_1	14·15	3·15	m	112	3·19
II_2	16·15	2·77	m	022, 122, 312	2·79, 2·76, 2·77
III_1	20·18	2·23	wm	003, 013	2·32, 2·25
III_2	21·95	2·06	ms	023	2·08
III_3	24·35	1·87	vw	033, 133, 423	1·87, 1·85, 1·84

$a = 15{\cdot}7$ Å $\quad b = 9{\cdot}44$ Å $\quad c = 6{\cdot}95$ Å

published work of Warwicker (1954) and of Marsh, Corey & Pauling (1955*a*,*b*); it should be noted, however, that a simple transformation of axes is necessary to relate the work of the latter authors to the scheme of indexing proposed here.

5. Discussion

The classification of the fibroins with an a-dimension of 10·6 Å into two groups (3a and 3b) calls for some discussion. This classification has been carried out on the basis of visual comparison of the density of the two main diffraction arcs E_2 and E_3, which assumes that the eye gives a measure of the integrated density. In single-crystal work such an assumption is generally reasonable when comparison is made between standard and unknown spots, which are not only small but of approximately the same size. For fibre work, however, where reflexions are usually large, this assumption is probably invalid, and a measure of the integrated intensity should be obtained in a more objective manner.

An attempt to clarify this situation was made by taking diffractometer scans across the equator and around the arcs of the reflexions E_2 and E_3 of the diffraction patterns for the fibroin of *Antheraea mylitta* (group 3a) and *Dictyoploca japonica* (group 3b). The resultant equatorial scans are shown in Fig. 1, where it is seen that the height of E_2 is certainly lower than that of E_3 for the fibroin of *Dictyoploca japonica*, in accordance with the visual estimation.

If the envelope of the equatorial scan is decomposed into its component curves it is found that the ratio of the integral half-breadths of $E_2 : E_3$ is 1·51 : 1 for *Dictyoploca japonica* and 1·28 : 1 for *Antheraea mylitta*, that is, the E_2 reflexion of the fibroin of *Dictoyploca japonica* is broader relative to E_3 than the corresponding reflexions of the fibroins of *Antheraea mylitta*. If now the ratio of the areas under the curves $E_2 : E_3$ is taken it is found to be 1·11 : 1 for *Dictyoploca japonica* and 1·33 : 1 for *Antheraea mylitta*, that is, for both fibroins the area under the peak E_2 is greater

than that under E_3, despite the misleading visual estimation of the density of the arcs on the X-ray diagrams of the fibroin of *Dictyoploca japonica*. Such an estimate of area, however, does not give a true measure of the integrated intensity because no account has been taken of the orientations of the reflexions. Allowance for orientation was made by multiplying the areas under the peaks E_2 and E_3 by the corresponding integral half-breadths of the azimuthal scans, the resultant value being then taken as proportional to the integrated intensity of the reflexion. The ratio of the integrated intensities E_2 : E_3 found in this way was 1·08 : 1 for the fibroin of *Dictyoploca japonica* and 1·29 : 1 for the fibroin of *Antheraea mylitta*. It will be seen by comparison with the previous ratios of the areas that the effect of orientation is small because E_2 and E_3 are approximately equally oriented in the diffraction patterns of the two

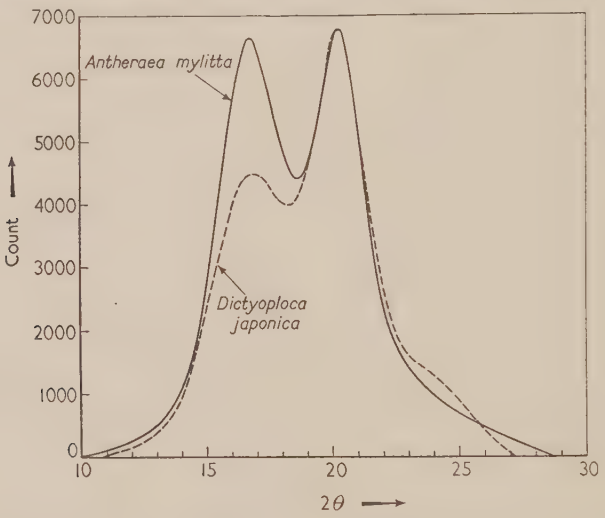

Fig. 1. Equatorial diffractometer scan of fibroins in groups 3a and 3b.

fibroins. The ratio of the integral half breadth of the azimuthal scan of E_2 (*Dictyoploca japonica*): E_2 (*Antheraea mylitta*) is 1·72 : 1 and the corresponding ratio for the E_3 reflexions is 1·70 : 1. Since a measure of the over-all orientation can be taken as the inverse of the integral half-breadth, these figures show that the crystallites in the fibroin of *Dictyoploca japonica* are less oriented than those in *Antheraea mylitta*.

As a result of this analysis it would seem that there is still some difference between these two fibroins representing the two groups 3a and 3b. This difference is mainly shown by the decreased intensity of E_2 relative to E_3 for the fibroin of *Dictyoploca japonica* compared with the corresponding integrated intensities found with the fibroin of *Antheraea mylitta*. This suggests that there is an electron deficiency in the (200) planes of the crystallites of the fibroin of *Dictyoploca japonica* compared with the corresponding planes of the crystallites of *Antheraea mylitta*. Since the composition of the (200) planes is greatly affected by the side-chain distribution in the fibroins, this difference suggests that there might be a different distribution of side-chains in groups 3a and 3b fibroins. Furthermore such a difference might lead also to different degrees of order in the packing and orientation of the crystallites of these two groups of fibroins. However, examination of the X-ray diagrams of all the fibroins of these two groups suggests that there is a gradation of properties, and strictly group 3b should be described as a sub-group of group 3a rather than as a distinct group.

An investigation into the difference between the X-ray diagrams of the fibroins in groups 2a and 2b has not yet been carried out, but it is probable that similar results will be found to those just described for fibroins in groups 3a and 3b.

Although visual examination of the X-ray diagram is sufficient to show the possibility of differences in structure it should be emphasized that for the purpose of quantitative structural determination visual estimation of relative intensity can be not only misleading but also quite erroneous.

This study of the extended range of fibroins has revealed another important fact. The fibroins in groups 4 and 5 have crystalline regions whose unit cells have a-dimensions of 15 and 15·7 Å respectively. Such large a-dimensions suggest that amino acids with long side-chains fit into the crystalline regions. Reference to the chemical analysis (Lucas, Shaw & Smith, 1960) of these fibroins supports this supposition because they have much lower contents of glycine and alanine, but much higher contents of amino acids with long side-chains, than the other fibroins. Conversely, it might be inferred that although the fibroins of groups 1, 3a, and 3b contain up to 20% of amino acid residues with long side-chains, these residues are unlikely to be included to any appreciable extent in the crystalline regions. It is therefore thought to be more probable that, in the fibroins of groups 1, 3a, and 3b, those portions of the polypeptide chains containing residues with long side-chains are to be found in non-crystalline regions, and mainly those portions of the chains with residues with short side-chains are included in the crystalline regions.

The chemical evidence also shows that, if the residues of glycine, alanine, and serine are regarded as those with short side-chains, and all the rest of the residues as those with long side-chains, then the fibroins of groups 4 and 5 contain about equal amounts of both types of residue. Since it is proposed to include both types of residue in the crystalline regions as well as in any non-crystalline regions, the simplest assumption to make is that the two types remain in equal proportions throughout. Such an assumption makes it simpler to discuss possible structures for the fibroins of groups 4 and 5, but it can be shown that some of the proposed structures can be extended to include those in which this assumption is not necessary.

The general type of packing of the anti-parallel pleated sheets in the unit cells can be derived from the fact that the X-ray diagrams of all the typical fibroins show strong 120 reflexions. Marsh, Corey & Pauling (1955*a*) attribute such a strong reflexion to the staggering of the sheets in relation to each other in the b-direction (their a-direction) with the result that the side-chains of one sheet project towards the space between the backbone chains in the next sheet. This feature, therefore, seems common to all the fibroins, but does not wholly determine the packing of the sheets, which will be shown to depend also on the sequence of the amino acid residues in the polypeptide chains composing the sheets.

If it is assumed that there is a regular alternation of amino acid residues with long and short side-chains then two basic structures can be proposed as shown in Fig. 2. In the first structure sheet B is turned through 180° relative to sheet A and may be shifted any distance in the direction of c consistent with the requirements for the establishment of minimum energy positions. As shown diagrammatically the structure would have twofold screw axes parallel to c, but this is not a necessary feature of this structure. The packing distances of the sheets from one another would be unequal : distance a_1 would be determined by the number and position of the short side-chains, distance a_2 by the long side-chains. This structure exists in simpler form in the fibroin of *Bombyx mori* (group 1) where the 'short side-chain' is the hydrogen of glycine and the

'long side-chain' is the methyl group of alanine. If a_1 is determined by the longest side-chains—methyl and hydroxymethyl—present in this region then this distance will be approximately 5·3 Å, the distance between sheets in β-poly-L-alanine or in the fibroin of *Antheraea pernyi*. Distance a_2 must then be 9·7 Å for group 4 and 10·4 Å for group 5 fibroins, which would be adequate for the inclusion of most long side-chains found in fibroins. The fibre-axis repeat distance would be 7 Å providing $L_1 = L_2$.

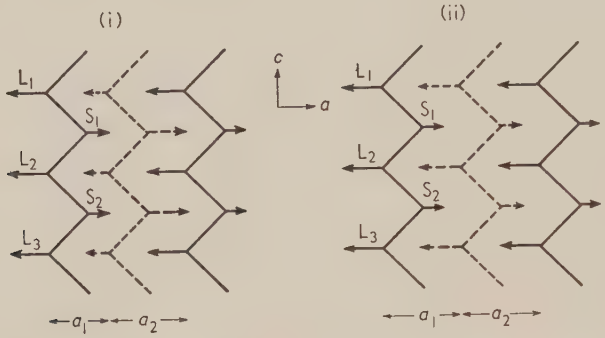

FIG. 2. Structures with regular alternation of amino acid residues with long and short side-chains (sheet B is displaced in the *b*-direction relative to sheet A).

The second structure of this type is simply the packing of sheets all oriented in the same sense. Again sheet B can be shifted any distance in the *c*-direction as steric requirements and other factors demand, so the arrangement shown is only schematic and must not be interpreted too rigidly. Distances a_1 and a_2 will be approximately equal in this structure and therefore must be 7·5 Å for group 4 and 7·85 Å for group 5 fibroins. These distances may be too small to permit the presence of amino acid residues with the longest side-chains in the crystalline regions, unless these side-chains can fold sufficiently to fit the space allowed. The axial repeat distance again would be 7 Å in the simplest case.

In these structures, and in those that follow, it must be emphasized that the side-chain of L_1 need not be the same as those of L_2, etc., and similarly for S_1, S_2, etc.; and hence any apparent symmetry displayed by these diagrams is not necessarily present. Furthermore the variable lengths and chemical properties of the side-groups introduce uncertainties in the exact distance between the sheets which might vary along the length of the crystallite, the X-ray diagram being capable of giving only the mean distance.

A second group of structures can be derived on the supposition that the sequence of amino acid residues is LL SS LL . . . where L and S stand for residues with long and short side-chains respectively. The basic pattern of these structures is shown in Fig. 3. As shown the chains are symmetrical and there would be no apparent advantage in a second basic structure with B rotated through 180° relative to A. However, as before, it must be emphasized that since L_1, L_2, etc., S_1, S_2, etc., differ, such a structure in fact is different. Chain B is only shown diagrammatically in the same vertical position as A; it can be shifted vertically into any position demanded by the other factors of the structure. It is clear that larger runs of amino acids of the same type give virtually the same structure, so that the assumption of equal proportions of amino acid residues with long and short side-chains is not really necessary. If

L = S = alanine, this structure reduces to that proposed by Marsh, Corey & Pauling (1955*b*) for the crystalline regions of *Antheraea pernyi* (group 3) and β-poly-L-alanine.

The inter-sheet distances in these structures should be approximately equal, that is, 7·5 Å and 7·85 Å for fibroins of groups 4 and 5 respectively. These distances are sufficient for the introduction of the side-chains of most of the amino acid residues found in fibroins, although it might be doubtful whether the side-chains of tyrosine, phenylalanine, histidine, and arginine can be easily accommodated. In the fibroins of groups 4 and 5 the number of these latter residues is small and whether they can fit into the crystalline regions therefore becomes only of small importance.

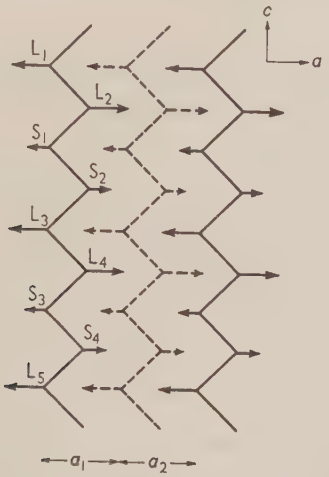

FIG. 3. Structures with repeating runs of amino-acid residues with long and short side-chains (sheet B is displaced in the *b*-direction relative to sheet A).

The fibroins of groups 4 and 5 contain relatively large proportions of the residues of serine, glutamic acid, and aspartic acid. If these residues occur in the crystalline regions, as is likely, then the structures just discussed allow a new feature to appear in the structure of fibroins, namely the possibility of inter-sheet bonding brought about by the hydrogen-bonding of groups in the side-chains. Chemical evidence seems to support this suggestion, especially for some fibroins of group 4, where it is found that these fibroins swell, and that from *Thaumetopoea pityocampa* dissolves, in mild aqueous alkalis (Shaw & Smith, 1960), a property not found with other fibroins.

The true fibre-axis repeat for these structures as shown should be 14 Å, but this requires L_1 to equal L_3, etc. However, this need not be true in an actual structure and then it becomes obvious that any figure in terms of a simple unit cell for the axial repeat distance becomes virtually meaningless for any of the structures discussed. The true repeat could be easily the complete polypeptide chain and this might be distributed between crystalline and non-crystalline regions.

Similar remarks apply to the *a*-direction because it is not established that every third sheet is in the same position with regard to the amino acid residue sequence as the first sheet, as is assumed in these structures. An infinite variety is possible when such details are taken into account, and it is also possible that no true repeat occurs in this direction at all.

Structures given in terms of unit cells can therefore be regarded only as convenient abstractions for the purpose of description. If the concept of the unit cell is abandoned then the crystalline regions must be regarded as networks of three-dimensional order

with only apparent repeat sequences. To obtain true structural details a complex system of atomic parameters would then be required. Such a system would render tedious the computation of theoretical intensities, but it would appear that the use of optical analogues might offer some solution to the difficulty.

Although some structural details have been given for fibroins of the groups 1, 3a, 3b, 4, and 5, none has been given for *Anaphe moloneyi*, a fibroin of group 2a. This fibroin contains over 90% of glycine and alanine residues in almost equal proportions (Lucas, Shaw & Smith, 1955), and would seem to be an almost ideal structure. The unit-cell dimensions differ only slightly from those of the fibroin of *Bombyx mori*, and apparently the contents of the crystalline regions are also similar. If the first general structure given in Fig. 2 was typical of the fibroin of *Bombyx mori*, then it was thought that the difference found with the fibroin of *Anaphe moloneyi* might be due to its having the second structure of Fig. 2. Work on this assumption did not meet with much success and it would seem that the structure of the fibroin of *Anaphe moloneyi* must fit one of the other general structures described. Preliminary chemical results (Lucas, Shaw & Smith, 1956) show that the fibroin of *Anaphe moloneyi* has some alanyl-alanyl links, so that the structures of the type shown in Fig. 3 seem to be more probable. Thus if in the structure shown in Fig. 3, $L_1 = L_2 = L_3$ etc. = alanine residues, and $S_1 = S_2 = S_3$ etc. = glycine residues, the fibre axis repeat distance should be 14 Å. If now sheet B is shifted by half the length of the unit cell in the direction of the fibre axis with or without rotation through 180°, then only $00l$ reflexions with $l = 2n$ would occur (Ellis & Warwicker, 1958), the first of which would correspond to a spacing of 7 Å. Such a reflexion is recorded on the X-ray diagrams of the fibroin of *Anaphe moloneyi* but this alone cannot prove any particular structure to be present. However, the recovery of alanyl-alanine and similar larger peptides from partial hydrolysates of the fibroin of *Anaphe moloneyi* is relatively low and therefore the number of alanyl-alanyl links in the fibroin may not be sufficient to permit extended regions of the type discussed. The structure of the fibroin of *Anaphe moloneyi* is therefore still open to speculation.

It appears from this discussion that the general structures shown in Figs. 2 and 3 can be modified to fit the structures of the crystalline regions in all the fibroins so far examined. It is evident that, contrary to what was first thought, no fibroin has a simple structure, and that the nature of the non-crystalline regions is as yet largely unknown. Further work is in progress to try to clarify the structural features of these fibroins.

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J. Mol. Biol. (1960) **2**, 363-371

Base Pairing in DNA

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On the basis of recent structural information which extends somewhat the range of possible dimensions for bases and base pairs, five new geometrically acceptable hydrogen-bonded base pairs which might be formed by the bases in nucleosides have been suggested. The dimensions of one of these new pairs, adenine-guanine, are such that this pair would apparently fit in the Watson-Crick DNA helix.

The evidence cited by Spencer against the possible existence in DNA of a pairing adenine-thymine guanine-cytosine which differs from that formulated by Watson & Crick has been examined and is considered to be inconclusive. Particular attention has been given to the conformational relationship between the base and the sugar, and a torsional angle, ϕ_{CN}, has been defined. This is the angle between the trace of the plane of the base and the projection of the C—O bond of the furanose ring when viewed along the glycosidic C—N bond. Values of this angle in known structures and proposed models have been tabulated, and the implications concerning possible conformations are considered.

1. Introduction

In a survey (Donohue, 1955, 1956) of the number of ways in which the four bases of DNA can form hydrogen-bonded pairs with one another, twenty-four different possible pairings were described, and it was pointed out that eight of these had already been observed in crystals of purines, pyrimidines, or related compounds. In addition, two of the pairings have since been suggested as a possible basis for the structure of polyriboadenylic acid: Morgan & Bear (1958) formulated, but did not favor, a structure based on pairing No. 2, while Watson (1957) constructed a model based on pairing No. 1. Fresco (1959) has recently indicated that the latter structure is to be preferred to that of Morgan & Bear.

Because at that time precise X-ray crystallographic information on compounds of the type in question was meagre, a minimum number of reasonable assumptions was made in defining the models used in the survey. The models used had rings with regular pentagons and hexagons with sides equal to 1·36 Å, and external C—NH_2 and C=O bonds of 1·36 and 1·21 Å, respectively. The hydrogen bond distances N—H . . . O and N—H . . . N were taken as 2·85 and 3·00 Å, respectively, and a maximum deviation of N—H . . . X from linearity of about 15° was allowed. In

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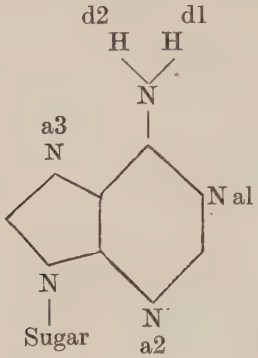
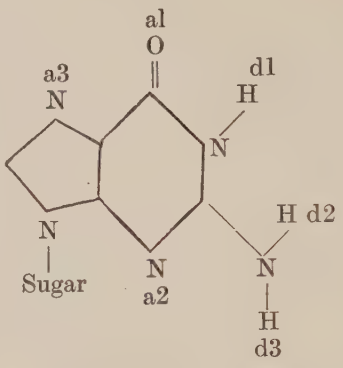
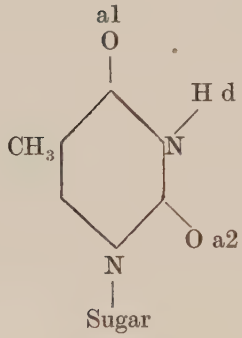
describing the base pairs, the hydrogen-bonding donor and acceptor sites were numbered as follows:



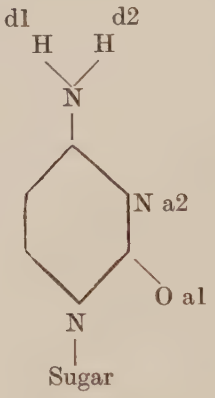
Adenine (A)



Guanine (G)



Thymine (T)



Cytosine (C)

Such structural information as was available was used shortly afterwards by Pauling & Corey (1956) to propose dimensions for the four bases which differed significantly from those used in the survey. On the other hand, their conclusion that it was possible to form the pairs adenine-thymine and guanine-cytosine such that these could be inserted in either order into a complementary two-chain helical structure for DNA did not differ from that arrived at earlier by Watson & Crick (1953), and in the survey. (These pairs are Nos. 5 and 15.) Utilizing new structural information which had since been published, Spencer (1959*a*) recently formulated a second set of dimensions for the bases which differ significantly from those proposed by Pauling & Corey. However, again the conclusion (Spencer, 1959*b*) regarding the compatibility of the A-T G-C pairing with the two-chain helical structure for DNA was unchanged.

2. Additional Base Pairs

The dimensions of the models and the restrictions on the geometry of the hydrogen bonding that were used in the survey were such that a mode of pairing for adenine and thymine recently discovered by Hoogsteen (1959) lies outside the limits originally set. (The original models give, for N—H . . . O linear, a deviation of about 25° for N—H . . . N, or, for N—H . . . N linear, a deviation of about 25° for N—H . . . O.)

It therefore appears that in forming base pairs the requirement of strict colinearity of the atoms N—H . . . O, whilst allowing deviations of up to 15° in the atoms N—H . . . N, is too stringent, although in the structure reported by Hoogsteen the geometrical requirements of the other hydrogen bonds not relevant to the pairing may be the cause of the relaxation of the conditions mentioned above. On the other hand, it is possible that expected departures from the assumed regularity of the five- and six-membered rings, inequalities in the external bond angles, or other differences from the models may allow the formation of satisfactory hydrogen bonds in this structure, or that deviations of more than 15° are found. Clarification of this point must await the completion of the structure refinement. It may well be that if pairing No. 5 (Watson-Crick) were used as the basis of the structure, then the formation of a satisfactory hydrogen bond by the other hydrogen atom of the amino group of adenine (d2) would be impossible, or would be at least distinctly less favorable than the observed arrangement. In any case, allowance of heptagonal hydrogen-bond systems, as in Fig. 1, together with variations of the bond angles and distances from the mean values previously used, and with only small deviations of N—H . . . X from linearity, extends the list of possible hydrogen-bonded base pairs to twenty-nine, as shown in Table 1, in which the notation of the survey is retained. (Five additional pairs which can form only with unsubstituted bases are not included in the list, since they cannot be formed by nucleosides. No. 29 may possibly also belong in this category.)

TABLE 1

Additional geometrically acceptable base pairs in polynucleotides

Base I Base II	Hydrogen bonding I-II	I-II
25. Adenine-Thymine	d2-a1	a3-d
26. Adenine-Thymine	d2-a2	a3-d
27. Adenine-Cytosine	d2-a2	a3-d2
28. Adenine-Guanine	d2-a1	a3-d1
29. Adenine-Guanine	d2-a2	a3-d3

No. 25 is the one observed by Hoogsteen in the 1 : 1 complex of 9-methyladenine with 1-methylthymine.

Noteworthy is the fact that pairing No. 28, shown in Fig. 1, may be inserted, in either order, into the Watson-Crick structure for DNA, with only very small "distortions" from the dimensions of the bases, whether those given by Pauling & Corey or Spencer, or, for that matter, from the cruder values used by Crick & Watson (1954). The dimensions shown in Fig. 1 should not be regarded as definitive. We wish merely to show that a pair having acceptable dimensions can be constructed. The average deviations of the dimensions given in Fig. 1 from those given by Pauling & Corey and Spencer, respectively, are 0·018 Å and 3·0°, and 0·015 Å and 3·6°. On the other hand, the average differences between the Pauling-Corey and Spencer dimensions are 0·024 Å and 3·3°. For one angle in adenine (C_4—C_5—C_6), the Pauling-Corey and Spencer dimensions differ by 15°. We have used a value closer to that of Spencer, but call attention to this difference to illustrate how rather widely varying predictions have been made on the basis of very nearly the same sets of experimental data, and

not to express a preference for one or the other. The difficulties in making accurate predictions of the dimensions of the various features of DNA at the present time are naturally just as important for construction of the pair of Fig. 1 as for construction of the usual Watson-Crick pairs. If the dimensions of pair No. 28 are not incompatible with the details of future X-ray crystallographic studies, then discussions of the complementarity of the Watson-Crick structure for DNA *per se* (rather than of the specificity of the enzymatic reactions by which DNA is made) must be suitably modified.

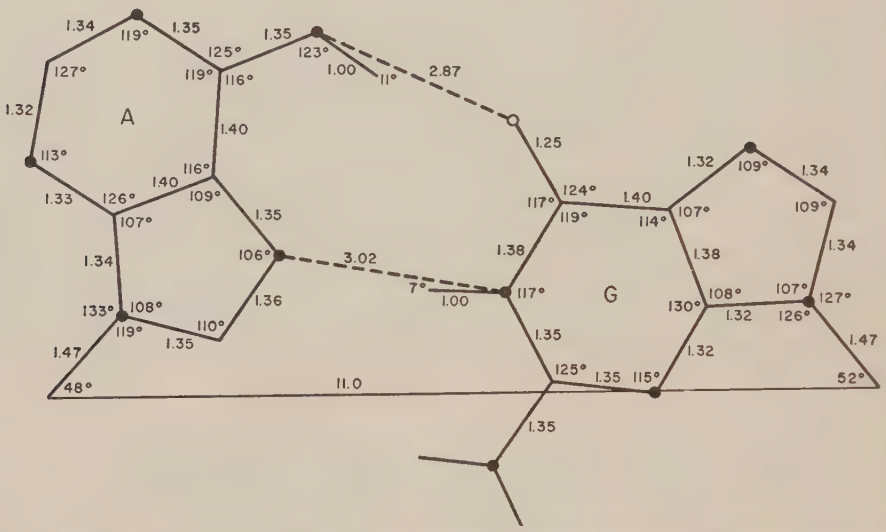


FIG. 1. Base pairing No. 28. The filled circles represent nitrogen atoms; the open circle represents an oxygen atom.

It is perhaps of interest to inquire into the nature of the "distortions" referred to above. Both Pauling & Corey and Spencer, for example, assumed that the external bond angles at a given atom in the 5- and 6-membered rings were equal (such as angles N_1—C_6—N and C_5—C_6—N in adenine, and C_5—C_6—O and N_1—C_6—O in guanine, etc.). Most, if not all, of the "strain" in fitting pair No. 28 into the Watson-Crick scheme can be relieved by the use of molecular coordinates derived from models of the bases having unequal external bond angles, a procedure which is in fact indicated by the available data. Observed inequalities in external bond angles range from as much as $21 \pm 11°$ and $10 \pm 11°$ in a cyclonucleoside (Zussman, 1953), to $20 \pm 9°$ in guanine hydrochloride (Broomhead, 1951), $11 \pm 2{\cdot}6°$ and $10 \pm 2{\cdot}6°$ in calcium thymidylate (Trueblood, Horn & Luzzati, 1960), $12 \pm 2{\cdot}2°$ and $8 \pm 2{\cdot}2°$ in 5-bromo-4,6-diamino-pyrimidine (Clews & Cochran, 1949), $10 \pm 4°$ in histidine hydrochloride (Donohue, Lavine & Rollett, 1956), $7{\cdot}8 \pm 0{\cdot}7°$ in thymine (Gerdil, 1960), $8 \pm 2°$ in caffeine (Sutor, 1958), $8 \pm ?°$ in 2-amino-4,6-dichloropyrimidine (Clews & Cochran, 1948), and $7 \pm 2°$ in adenine hydrochloride (Cochran, 1951). The degree of precision in the above studies is seen to vary considerably. The stated uncertainties were calculated from the standard errors as estimated (and perhaps often under-estimated) by the respective authors. Nevertheless, it appears that the assumption of equal external bond angles is not necessarily valid, a situation not entirely unexpected both because of the lack of the requisite symmetry in the resonating heterocyclic systems under examination, and because of the ease of deforming bond angles. It is of interest that

the dimensions (including the unequal bond angles) for guanine given in the old and imprecise determination were those used in some of the earlier formulations of Watson-Crick models of DNA for subsequent testing of the structure against the X-ray data (e.g., Crick & Watson (1954) and Wilkins (1957)).

The experimental data just cited emphasize the pitfalls inherent in any procedure which involves the use of averaged or idealized molecular dimensions to arrive at standard and limiting dimensions. In another example, Spencer (1959*a*) assigned the value 120° to the angle N_3—C_2—O_2 in pyrimidines, and in his discussion (1959*b*) of a possible structure for deoxycytidine referred to a "distorted model" in which this angle is 124°. In fact, the *observed* values of this angle in uracil (Parry, 1954), thymine (Gerdil, 1960), and the thymidylate ion (Trueblood *et al.*, 1960) are respectively 124·5°, 122·7°, and 123°. Although Spencer's standard molecular dimensions represent an improvement over any earlier values available, there are, nonetheless, significant individual discrepancies of as much as 0·05 Å in a bond distance and 7° in a bond angle for his thymine model when compared with the observed dimensions in thymine and the thymidylate ion. Spencer was careful to emphasize the difficulties and uncertainties in deriving his "probable structures of the bases" (1959*a*) and "limiting dimensions of the base pairs" (1959*b*), but it appears that his estimates of his accuracy (0·02 Å in bond distance and 4° in bond angles) were over-optimistic. Errors of this sort in turn introduce errors and uncertainties in the limiting dimensions and in the estimates of the distortions that can be tolerated by a molecule.

3. The Alternative Pairing

In his second paper on DNA, Spencer (1959*b*) again confirmed an earlier observation (Donohue, 1956): that there exists a structurally acceptable pairing A—T G—C (Nos. 4 and 17) which is different from that proposed by Watson & Crick (1953). Spencer further stated with regard to these alternative pairs that "one cannot be sure on X-ray evidence alone that they might not form the basis of the structure of DNA" but added "there is strong indirect evidence that they do not, in fact, occur in DNA, but it has proved impossible to reject them altogether. Further work on molecular models will be necessary if the question is to be settled conclusively. There is meanwhile no reason to doubt that the Watson-Crick pairing scheme is correct."

The "strong indirect evidence" cited by Spencer falls into three categories: (i) observations on rates of enzymatic synthesis of DNA *in vitro* with different deoxynucleoside triphosphates; (ii) evidence for a twofold axis perpendicular to the molecular axis of DNA; and (iii) evidence against the existence of different conformations about the glycosidic C—N bond. The evidence in the first two of these categories seems to us at best very weak. The experiments on rates of synthesis, originally available only in abstract form, have now been described more fully (Bessman *et al.*, 1958), and it is clear that the rates observed reflect a great many different factors. In the words of the authors "it is not feasible at this time to attribute different rates of incorporation of (the different) deoxynucleoside triphosphates to conditions controlled simply by the hydrogen-bonding characteristics of the base pairs" and "the specificity of the enzyme toward a given substrate must be considered, as well as the unexplored influence of the DNA primer." With regard to the evidence for the dyad axis perpendicular to the DNA chains, Spencer himself has pointed out that the phosphate groups alone (which could be related by a dyad axis in the alternative pairing) might dominate the packing sufficiently to lead to crystallinity. Furthermore,

although the fact that two interaxial angles are close to 90° might be suggestive of monoclinic symmetry, it is well known that many triclinic crystals have two or three interaxial angles close to 90°; in a recent compilation (Donnay & Nowacki, 1954), 45% of all triclinic crystals listed have two interaxial angles equal to 90 ± 5°, and in one-fourth of these the angles are within 1° of orthogonality.

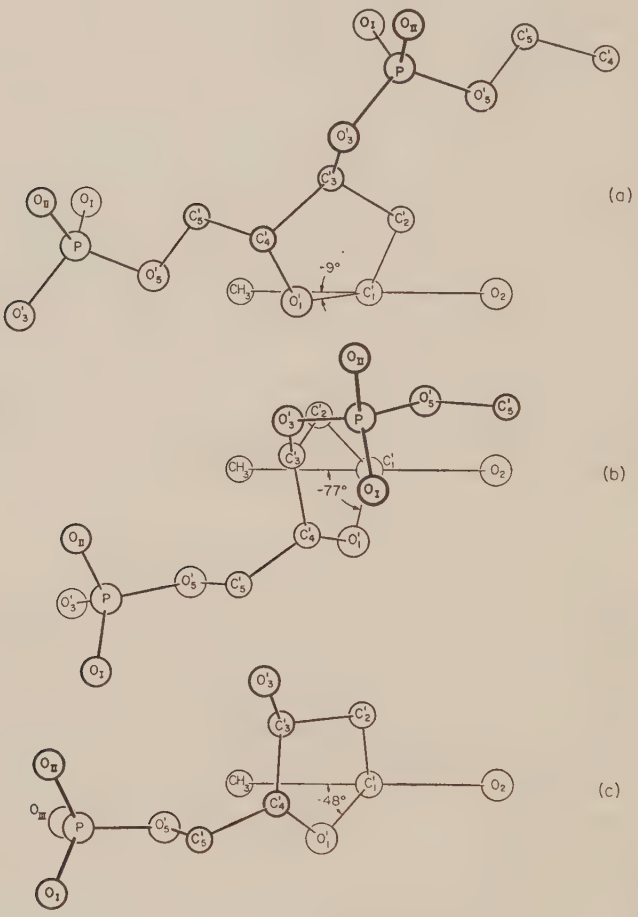


FIG. 2. The conformation about the glycosidic C—N bond and the torsional angle, ϕ_{CN}, in:
(a), the structure of DNA according to Crick & Watson (1954);
(b), the structure of DNA according to Langridge *et al.* (1957);
(c), the thymidylate ion (Trueblood *et al.*, 1960).
The base illustrated in each instance is thymine.

The evidence regarding conformations about the glycosidic C—N bond is worthy of more detailed consideration. The X-ray diffraction data for DNA were said to suggest strongly that the sugar-phosphate chains are not related by a twofold axis coincident with the molecular axis (Crick & Watson, 1954). Thus, as Spencer indicates, it is likely that the two chains are anti-parallel, and indeed recent chemical evidence strongly supports this conclusion (Josse & Kornberg, 1960*a*,*b*); in the alternative pairing this results in two different conformations of the sugar relative to the base; these two are related by a rotation of 180° about the glycosidic (C_{sugar}—N_{base}) bond. Spencer states that only one of these has been observed in the crystalline pyrimidine

nucleosides so far studied, and concludes that this one "is stereochemically favourable compared with the other, and it is likely that DNA contains only this configuration."†

For convenience in discussing this conformational question we shall define the sugar-base torsion angle, ϕ_{CN}, as the angle formed by the trace of the plane of the base with the projection of the C—O bond of the furanose ring when viewed along the C—N bond. This angle will be taken as zero when the furanose-ring oxygen atom is *anti-planar* to C_2 of the pyrimidine or purine ring, and positive angles will be taken as those measured in a clockwise direction when viewing from C to N. Some typical angles are shown in Fig. 2 (Trueblood *et al.*, 1960). The striking difference between certain features of the original Watson-Crick model (Fig. 2(a)) and one of its latest modifications (Fig. 2(b)) is noteworthy.

Approximate values of ϕ_{CN} for the known nucleosides and nucleotides, as well as for certain model structures, are given in Table 2.

TABLE 2

Approximate torsion angles, ϕ_{CN}

Structures, deoxyribo-		
calcium thymidylate	− 48°	Trueblood, Horn & Luzzati (1960)
5′-Br-3′:5′-dideoxythymidine ‡	− 30° or + 7°	Woolfson (1956)
5′-Br-5′-deoxythymidine	− 65°	Huber (1957)
Structures, ribo-		
cytidine	− 20°	Furberg (1950)
cytidilic acid b	− 46°	Alver & Furberg (1959)
Models		
Watson-Crick, original	− 9°	Crick & Watson (1954)
Watson-Crick, modified	− 27°	Wilkins (1957)
Watson-Crick, remodified	− 77°	Langridge *et al.* (1957)
polyadenylic acid I	+ 153°	Morgan & Bear (1958)
polyadenylic acid II	+ 180°	Morgan & Bear (1958)
deoxycytidine, alternative	+ 126°	Spencer (1959*b*)

‡ Because of the ambiguities of a projection, the two possible angles for this compound cannot be distinguished.

Examination of molecular models of pyrimidine nucleosides suggests that there are two ranges of ϕ_{CN} within which relatively stable conformations might be assumed. Each of these ranges covers somewhat more than 90°, one being centered about *ca* − 30° (the *anti* range), and the other centered about *ca* + 150° (the *syn* range). These two ranges correspond to the two configurations referred to by Spencer. Although there are some very slightly closer contacts within part of the *syn* range, they do not appear to require any significant distortions; thus we agree with Spencer that models do not permit ruling out this conformation. The barriers *between* the two ranges appear to arise chiefly from steric interactions of one of the hydrogen atoms on C_2' of the sugar with the "ortho" substituents of the base. Without such direct steric interaction, the barrier might be expected to be relatively low because of the approximate analogy of these molecules with, for example, toluene and nitromethane, where the barrier to rotation about the bond between the trigonally and tetrahedrally

† The term "conformation" is to be preferred to "configuration" in discussions of possible rotational isomerism about single bonds; see, for example, Dauben & Pitzer (1956).

bonded atoms is only a few calories (Pitzer, 1955). Molecular models of adenylic and guanylic acids indicate that the steric hindrance to rotation about the glycosidic bond in these molecules may be significantly smaller than in the corresponding pyrimidine derivatives†. Indeed, the ease (Clark, Todd & Zussman, 1951) with which the cyclic adenosine nucleoside studied by Zussman (1953) is formed shows that at least in the absence of the phosphate group the *syn* conformation is readily achieved, for the cyclic compound has this conformation.

The data in Table 2 show that in the structures of the three deoxyribo derivatives (only those with pyrimidine bases have been studied) the torsion angle falls in the *anti* range, as Spencer pointed out; however, the angle varies widely within this range and it is clear that the potential function for rotation has a broad and shallow minimum. The specific conformation assumed thus depends rather sensitively on the balance of intra- and inter-molecular van der Waals and hydrogen-bonding forces. On the basis of the available evidence, it seems to us premature to conclude that conformations in the *syn* range are unlikely to occur because of some inherent instability, and it does not yet seem possible to deduce much about the barrier heights between the conformations. If it is true that for pyrimidines only conformations in the *anti* range exist naturally, the implications about the mechanism of their formation as well as about the height of the barrier between the two ranges should be investigated further.

4. Conclusion

The existence of an apparently acceptable pairing of adenine with guanine which fits into the Watson-Crick structure for DNA emphasizes again the need for additional structural studies on the various parts of the DNA molecule, since with the data now available it is not possible to assert that this pairing cannot become a part of the Watson-Crick DNA helix. If later work shows that in fact it can, then the particular complementary nature of the structure must be re-examined.

With regard to the possible role of the alternative A—T G—C pairing in the structure of DNA, the strongest evidence against it is that concerned with the observed conformations about the glycosidic C—N bonds in nucleosides and nucleotides, and even here the evidence does not seem conclusive, although this, and other indirect evidence, generally tends to favor the Watson-Crick pairing when the two structures are contrasted. We agree with Crick (1957) that more physico-chemical data are needed as a basis for judgment of models of nucleic acids, "for example, measurements of base pairing, tautomeric forms, van der Waals contacts, preferred orientations, and so forth."

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† Crick (1957) has pointed out that this is true for adenylic acid (in the DNA structure) but says that it is difficult if not impossible to get guanylic acid into what is here defined as the *syn* conformation because of the close contact of the amino group of guanine with the adjacent sugar. Our models do not show this close contact.

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Identification of the Abnormal Polypeptide Chain of Haemoglobin G_{Ib}

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The analyses of known haemoglobin mixtures in starch gel have been compared using borate, phosphate, and the discontinuous tris citrate-borate buffers. Hybrid haemoglobins formed during the neutralization of acid dissociated mixtures of appropriate haemoglobins may be detected using either alkaline phosphate or the discontinuous systems. The dissociation and recombination of Hb-G_{Ib} with Hb-S produces Hb-A and a doubly abnormal haemoglobin migrating near the position of Hb-C. Hb-J_{Tr} gives no new haemoglobin species when hybridized with Hb-S which has abnormal β-chains, but in contrast an additional haemoglobin zone appears in the position of Hb-A when Hb-J_{Tr} is hybridized with Hb-G_{Ib}. These experiments suggest that Hb-G_{Ib} has abnormal α-chains and that in the hybridization with Hb-J_{Tr} both Hb-A and another doubly abnormal haemoglobin whose net charge is similar to that of Hb-A are formed. Hb-G_{Ib} is therefore different from Hb-G_{Sa} which has abnormal β-chains. It is postulated that Hb-G_2 is the naturally occurring hybrid of Hb-G_{Ib} and Hb-A_2.

1. Introduction

Shooter, Skinner, Garlick & Barnicot (1960) have suggested as a result of a detailed electrophoretic examination that the abnormal haemoglobin which they describe is possibly identical with the original haemoglobin G (Edington, Lehmann & Schneider, 1955) but not with the haemoglobin (now called Hb-$G_{San\ José}$ or Hb-G_{Sa}) described by Schwartz, Spaet, Zuelzer, Neel, Robinson & Kaufman (1957). The chemical abnormality of Hb-G_{Sa} has now been shown to be in the β-polypeptide chains (Hill & Schwartz, 1959). The characterization of the abnormality in the haemoglobin of Shooter *et al.* (now called Hb-G_{Ibadan} or Hb-G_{Ib} for short) therefore provides a further basis for comparison of these haemoglobins and such a characterization is reported here. The identity of the abnormal polypeptide chain has been determined by the hybridization technique introduced by Itano & Singer (1958). This method depends on the fact that human adult haemoglobin (Hb-A), consisting of two pairs of polypeptide chains with a haem attached to each chain (Rhinesmith, Schroeder & Martin, 1958; Perutz, Rossmann, Cullis, Muirhead, Will & North, 1960), dissociates asymmetrically at pH's below 6·0 into two unlike subunits α_2^A and β_2^A. When the acid solution is subsequently neutralized, these subunits combine to re-form the Hb-A molecule $\alpha_2^A\beta^A$ (Itano & Singer, 1958). If two unlike haemoglobins are together dissociated and recombined, exchange of subunits may occur in which case hybrid

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haemoglobin molecules are formed. Such hybrid molecules are of the parent type if the two haemoglobins have a common pair of α- or β-chains (Singer & Itano, 1959). On the other hand, dissociation and recombination of two haemoglobins, one of which has abnormal α-chains and the other abnormal β-chains, produce Hb-A and a doubly abnormal hybrid haemoglobin as well as the parent types. If the original haemoglobins differ in net charge from Hb-A then the two new species may be distinguished from the parent haemoglobins and sometimes from each other by moving boundary electrophoresis (Itano & Robinson, 1959). Thus, when a haemoglobin with an unknown abnormality is hybridized with a haemoglobin known to have, say, abnormal β-chains, hybrids will be produced which are different from the parent types if the unknown haemoglobin has abnormal α-chains but not if it has abnormal β-chains. Itano, Singer & Robinson (1959) have in this way used sickle cell haemoglobin (Hb-S) which has aberrant β-chains (Vinograd, Hutchinson & Schroeder, 1959: Ingram, 1959) to show that their specimens of haemoglobins D, E and J have abnormal β-chains, whilst haemoglobins I and Hopkins-2 have abnormal α-chains.

Analysis of recombined mixtures of haemoglobins by moving boundary electrophoresis depends on the use of relatively large quantities of haemoglobin, which are not always available. This was so in the current investigation and it has prompted an examination of the analysis of haemoglobin recombination mixtures by zone electrophoresis on starch gel. Analyses of normal and abnormal haemoglobin mixtures in starch gel have already been reported by a number of workers. For example, Owen & Got (1957) and de Grouchy, Schapira & Dreyfus (1958), using borate buffer conditions essentially the same as those employed by Smithies (1955), were able to separate haemoglobins A, F, S, E and C. Clearer separation of these and other haemoglobins was later reported by de Grouchy (1958) using the discontinuous buffer system devised by Poulik (1957). Fessas & Mastrokalos (1959) have briefly commented that haemoglobins which migrate faster than Hb-A at alkaline pH may be differentiated in phosphate buffer of pH 6·5 and ionic strength 0·033, whilst those which migrate more slowly than Hb-A separate well in either the borate or the discontinuous buffer systems mentioned above. Huehns, Flynn, Butler & Shooter (1960) have found in addition that it is possible under carefully specified conditions in phosphate buffers to separate Hb-H and Hb-Bart's even at very low concentrations.

In order to determine which of these three buffer systems would be suitable for the detection of the hybrid haemoglobins, comparative analyses of a series of known haemoglobin mixtures have been made in all three buffers. These show that both phosphate buffer at specified pH and the discontinuous buffer system achieve wide separation of haemoglobins which differ by two units in net charge, and their subsequent successful use in the analysis of haemoglobin recombination mixtures is illustrated with hybridization experiments with haemoglobins G_{Ib}, S, and J. The combination of the hybridization technique and the starch gel analyses has now shown that, in contrast to Hb-G_{Sa}, HbG_{Ib} has abnormal α-chains.

2. Materials and Methods

Haemoglobin solutions. These were prepared by the method of Singer, Chernoff & Singer (1951) from fresh venous blood. The abnormal haemolysates used were from a case of Hb-H disease (Bingle, Huehns & Prankerd, 1958), haemoglobin-J trait (Huehns, 1960, unpublished observations), sickle cell trait, sickle cell-haemoglobin C disease, and Hb-G disease (Shooter *et al.*, 1960). All the experiments described were made with carbonmonoxyhaemoglobins. The latter were prepared by saturating the haemoglobin solution

with CO and adding solid sodium dithionite to reduce any methaemoglobin present. Subsequent operations were carried out under CO where possible and dialyses were made against the appropriate solution which had been previously saturated with CO.

Starch block electrophoresis. These experiments using maize starch (B.P.) obtained from British Drug Houses Ltd., Poole, England, followed the procedure due to Kunkel (1954). After separation of the haemoglobin zones had been achieved, the appropriate zone was cut out, placed on a sintered glass filter and the haemoglobin eluted from the starch with 0·02 M-NaCl. The haemoglobin was reconverted to carbonmonoxyhaemoglobin and the dilute solution concentrated in $\frac{1}{4}$ in dialysis tubing under vacuo in the cold. When the haemoglobin concentration had reached approximately 3 to 4 g % the solution was transferred to new tubing, sodium dithionite added if methaemoglobin was present, and then dialysed against two separate 1 litre volumes of 0·2 M-NaCl over a period of about 24 hr. Residual starch and small precipitates of denatured haemoglobin were finally removed by centrifugation.

Starch gel electrophoresis. Gels were prepared from "Connaught hydrolysed starch" obtained from Connaught Medical Research Lab., Toronto, Canada, by the method recommended by the manufacturers and were poured into perspex trays 22 cm long, 13 cm wide and 0·8 cm deep.

TABLE 1

Composition of solutions for phosphate buffer starch gels (pH measured at 25°C)

pH to which 0·04 M-$Na_2HPO_4.12H_2O$ was adjusted with H_3PO_4 [1]	pH of gel
8·1 [2,3]	7·7
8·1 [2]	7·4
7·8	7·1
6·8	6·8
6·2	6·3

[1] 40 ml. of this solution diluted to 300 ml. with H_2O used for making gel.
[2] No H_3PO_4 added to these solutions.
[3] Starch washed twice with this solution prior to making gel.

Borate buffer system. The composition of the gel and electrode buffers was that recommended by the manufacturers of the starch to give the best separation of the components of human serum. In this series one batch of starch was used and the buffers were as follows: gel buffer 0·021 M-boric acid and 0·0084 M-NaOH, and electrode buffer 0·30 M-boric acid and 0·06 M-NaOH. A constant current of 35 mA was passed for 4 hr.

Discontinuous buffer system. This was prepared according to the technique of Poulik (1957) using a 0·076 M-tris (2-amino-2-hydroxymethylpropane-1:3-diol)—0·05 M-citric acid solution for the gel and a 0·30 M-boric acid—0·06 M-sodium hydroxide solution for the electrode chambers and filter paper bridges. A constant current of 35 mA was passed for 2 to $2\frac{1}{2}$ hr.

Phosphate buffer systems. A gel made with 0·0053 M-$Na_2HPO_4.12H_2O$ (40 ml. of 0·04 M-$Na_2HPO_4.12H_2O$ diluted to 300 ml. with H_2O) had a pH of about 7·4. More alkaline gels were made with starch that had been washed prior to gelling with the above phosphate solution. A given weight of starch was allowed to stand for 16 hr with occasional stirring in the appropriate volume of phosphate solution. After settling, the supernatant was decanted and replaced by an equal volume of fresh phosphate solution. This process was repeated when the starch had settled. The gel was finally made by heating this solution; when cold it had a pH of 7·7. Gels more acid than pH 7·4 were made with solutions which had been diluted from 0·04 M-$Na_2HPO_4.12H_2O$ whose pH had first been adjusted with syrupy phosphoric acid B.P. (Table 1).

The electrode buffers for each experiment were made by adjusting 0·04 M-$Na_2HPO_4.12H_2O$ with acid to the appropriate final pH of the gel. A constant current of 50 mA was passed for from 2 to 5 hr.

After electrophoresis the gels were sliced and stained for protein with amido black 10B and for haemoglobin with either benzidine or *o*-dianisidine and H_2O_2 (Owen, Silberman & Got, 1958). They were subsequently photographed on Kodak Microfile film using green Wratten 58 and violet Wratten 39 filters respectively.

Hybridization experiments. The conditions for dissociation and recombination were in principle the same as those outlined by Itano & Robinson (1959) although much smaller volumes of solutions were used. It was found that the smallest volume which could be handled in the final dialysis was about 60 μl. and this was sufficient for two separate paper insertions in the starch gel. The lowest total haemoglobin concentration for satisfactory resolution of hybrid species was about 1% in the discontinuous buffer system and about $1\frac{1}{2}$% in the phosphate buffers.

Thus in a typical experiment 40 μl. of a 3% solution of Hb-G_{Ib} and 40 μl. of a 3% solution of Hb-S, both in 0·2 M-NaCl, were mixed and then divided into two equal volumes. To the first volume 40 μl. of ice-cold sodium acetate buffer of pH 4·7 and *I* 0·2 were added. After standing in an ice-water bath for 4 hr the solution was transferred with a Pasteur pipette to $\frac{1}{4}$ in dialysis tubing and dialysed in the cold against two separate one litre volumes of phosphate buffer of pH 6·8 and *I* 0·02 for a total of 20 hr. The second 40 μl. volume was used for the control and to it was added 40 μl. of the pH 6·8 phosphate buffer. It was then treated in exactly the same way as the acidified portion. Where the original haemoglobin solutions were less concentrated than 3% or when a phosphate buffer gel was to be used for the final analysis the equal volume of the acetate buffer of *I* 0·2 was replaced either with a one-tenth volume of an acetate buffer of the same pH but of *I* 2·0 or a proportionate volume of a buffer of intermediate ionic strength. No attempt was made to measure and adjust the concentrations of the hybridized and control solutions after dialysis. The former were usually the more dilute because of the precipitation of some haemoglobin during dialysis.

3. Results

(a) *Starch gel electrophoresis of standard haemoglobin mixtures*

In order to compare the resolution of different haemoglobins in the three buffer systems the following pairs of haemoglobin were analysed simultaneously: H + A, J + A, A + S and S + C. The difference in net charge between these abnormal haemoglobins and Hb-A in the pH range of the analyses in this series varies from about − 4 for Hb-H to + 4 for Hb-C, the difference in net charge being approximately two between haemoglobins J and A, A and S, and S and C (Hunt & Ingram, 1959*a*,*b*).

In the most alkaline phosphate buffer all the above haemoglobins migrated towards the anode and the separation of haemoglobins J and A, A and S, as well as S and C, was approximately the same (Plate I(a)). At this pH the Hb-H zone migrated too far and diffused too rapidly to be visible at the end of electrophoresis. In the early stages of this experiment, however, and of those at pH 7·4 and 7·1, the Hb-H zone could be clearly seen. At a gel pH of 7·4 (Plate I(b)) Hb-C migrated towards the cathode but the separation of the haemoglobins in the JA, AS, and SC mixtures was still approximately equal.

At the lower gel pH's, however, those zones which migrated appreciably towards the cathode became diffuse (Plate I(c), (d) and (e)). This, coupled with the decrease in the differences between the migration path lengths, largely obscured any resolution of the different haemoglobins. Thus at a gel pH of 6·3 there was no resolution of haemoglobins S from C and only poor and rather distorted separation of haemoglobins A from S and J from A. On the other hand the resolution of those haemoglobins which

still migrated towards the anode or only slightly towards the cathode at the lower pH's remained good (Plate I(c)).

The haemoglobin zones in the discontinuous buffer system were sharpened by the large potential gradient associated with the buffer boundary in the gel and although the actual separation of the zones under the chosen conditions was less than in the phosphate gels the overall resolution in the two systems was very similar (Plate II(a)). It was noted, however, that in the discontinuous system, unlike the phosphate system, the separation decreased for those haemoglobins migrating faster than Hb-A. Thus the distance between haemoglobins J and A was markedly less fast than that between haemoglobins A and S and moreover there was little difference in the migration path lengths of haemoglobins J and H.

The borate buffer system as used here gave an analysis comparable to the discontinuous system but with more diffuse zones (Plate II(b)). Attempts to analyse haemoglobin mixtures at varying pH but at constant sodium ion concentration in this buffer were not successful.

Hybridization experiments.

Hb-S has abnormal β-chains, since it differs from Hb-A by replacement of the glutamyl residues six residues from the N-terminal end of the β-polypeptide chains with valyl residues (Hunt & Ingram, 1959*a*,*b*). When Hb-G_{Ib} and Hb-S were together dissociated and recombined a new haemoglobin zone appeared near the position of Hb-C (Plate III(i)), and a zone of increased intensity in the position of Hb-A. This Hb-A zone is partially obscured by the foetal haemoglobin present in the Hb-S sample used in this experiment and which can be seen migrating ahead of both Hb-G_{Ib} and Hb-S in the control experiment.

Itano & Robinson (1959) have already found that there was no significant change in electrophoretic composition when a solution containing both Hb-S and Hb-J was acidified and re-neutralized and this has now been confirmed here by starch gel analysis using haemoglobin J (Hb-$J_{Trinidad}$ or Hb-J_{Tr}) from a different family (Plate IV). In contrast a new haemoglobin zone appeared in the position of Hb-A when Hb-G_{Ib} was hybridized with Hb-J_{Tr} (Plate V).

4. Discussion

In general satisfactory resolution of the haemoglobins in the mixtures used in this series (with the exception of Hb-H) was obtained in the discontinuous buffer system and in the higher pH phosphate starch gels. One advantage of the latter is that the distance the various haemoglobins migrate is directly proportional to the difference in net charge from Hb-A within the range from about -2 for Hb-J to $+4$ for Hb-C. Although little resolution of the haemoglobins which are more positively charged than Hb-A is achieved in the lower pH phosphate gels, the migration path length still remains roughly proportional to net charge for those haemoglobins which migrate towards the anode or only slightly to the cathode. It is for this reason that phosphate gels of pH 6·5 to 6·8 can be successfully used to differentiate Hb-H from Hb-A and Hb-J (Fessas & Mastrokalos, 1959; Huehns *et al.*, 1960). In contrast, in the discontinuous buffer system those haemoglobins which migrate faster than Hb-A separate less well. On the other hand the sharpness of the zones in the discontinuous system makes it especially suitable for the detection of haemoglobins present in low concentration, e.g. Hb-A_2. The separation of the haemoglobin zones in the borate buffer was the least satisfactory of the comparative experiments.

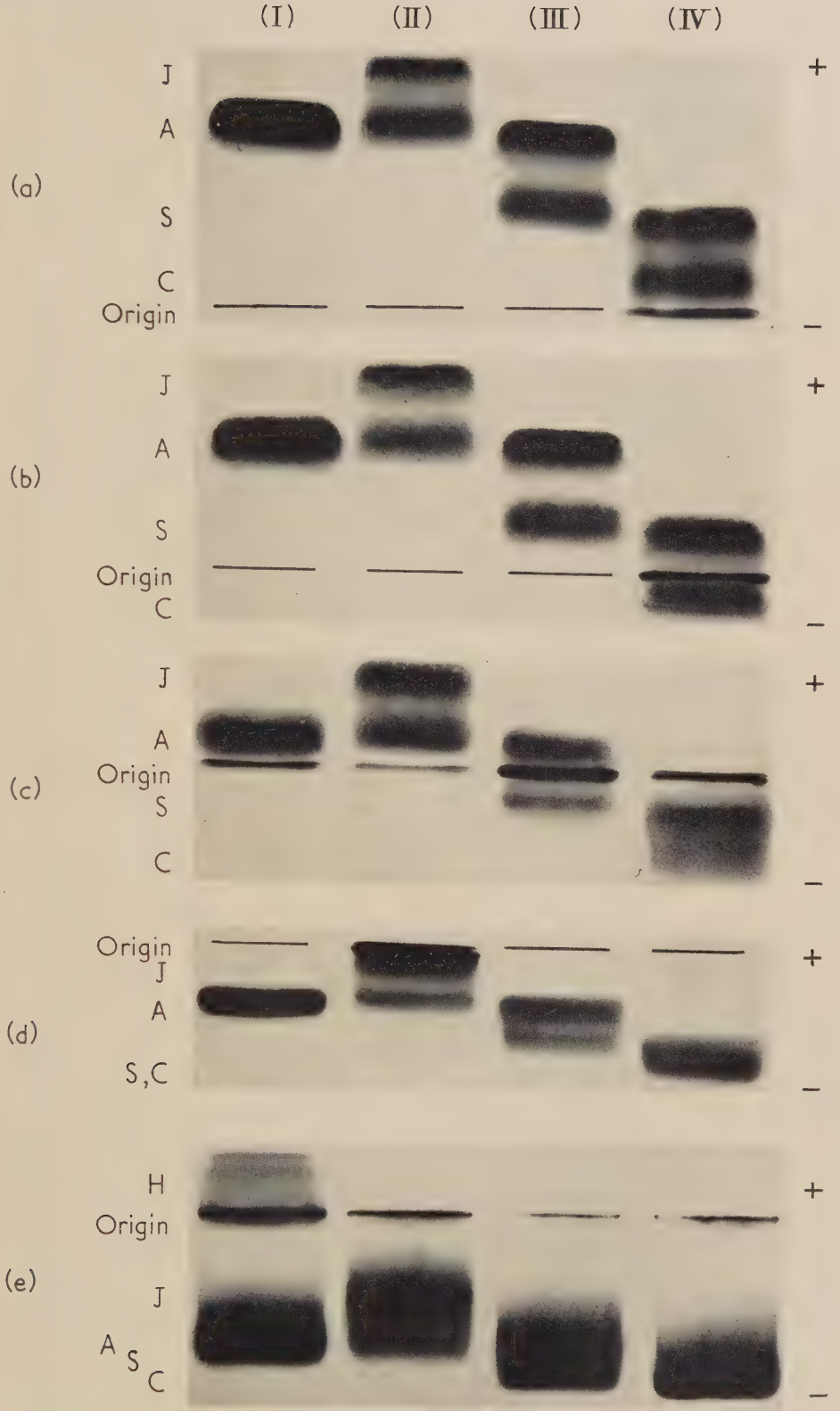

PLATE I. Starch gel analyses of haemoglobin mixture (I) H + A, (II) J + A, (III) A + S and (IV) S + C in phosphate buffer. Gel pH: (a) 7·7, (b) 7·4, (c) 7·1, (d) 6·8 and (e) 6·3. Migration time 5 hr, *o*-dianisidine stain.

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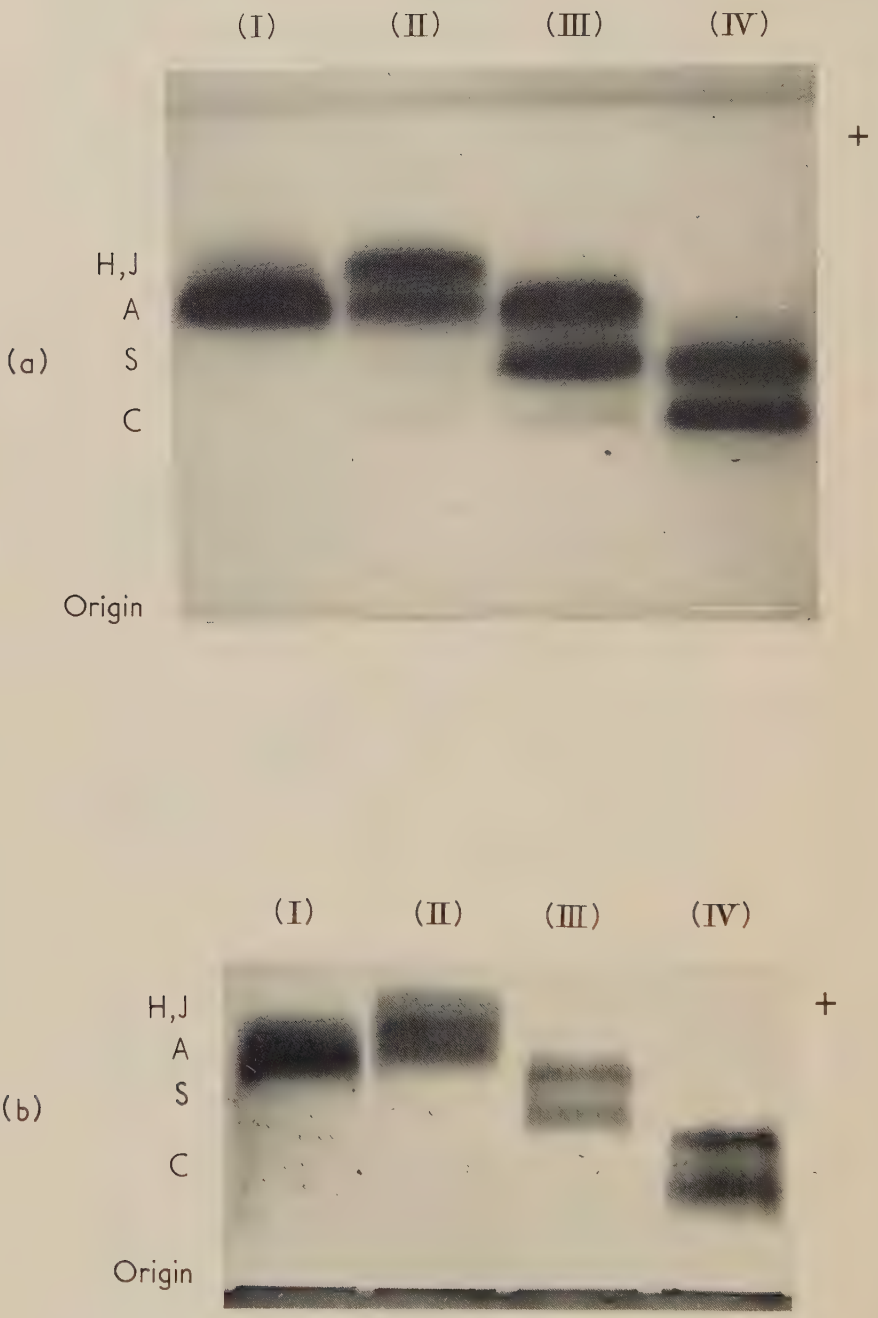


PLATE II. Starch gel analyses of haemoglobin mixture (I) H + A, (II) J + A, (III) A + S and (IV) S + C in (a) discontinuous tris citrate-borate buffer, gel pH 8·2; migration time 2 hr, *o*-dianisidine stain, and (b) borate buffer, gel pH 8·2; migration time 4 hr, *o*-dianisidine stain.

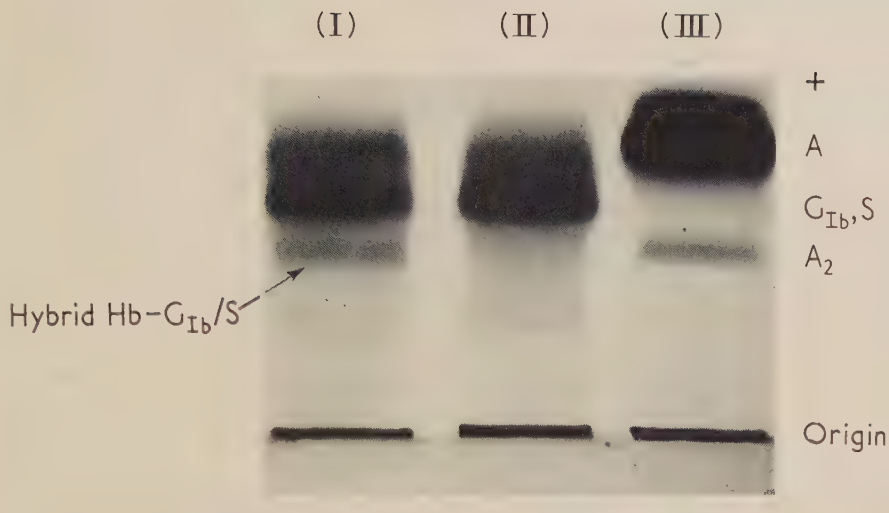
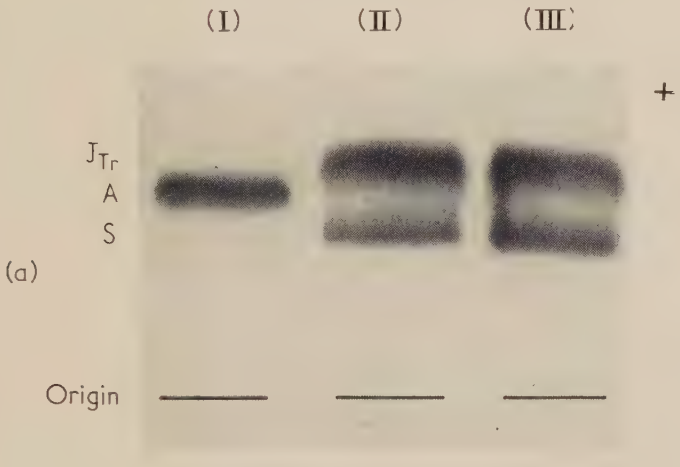


PLATE III. Hybridization of haemoglobins G_{Ib} and S. Starch gel analysis in the discontinuous system. Migration time 2½ hr, benzidine stain. (I) mixture of Hb-G_{Ib} and Hb-S dissociated and recombined, (II) untreated mixture of Hb-G_{Ib} and Hb-S, (III) normal adult haemolysate.



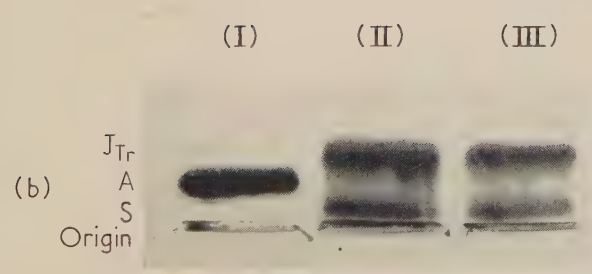
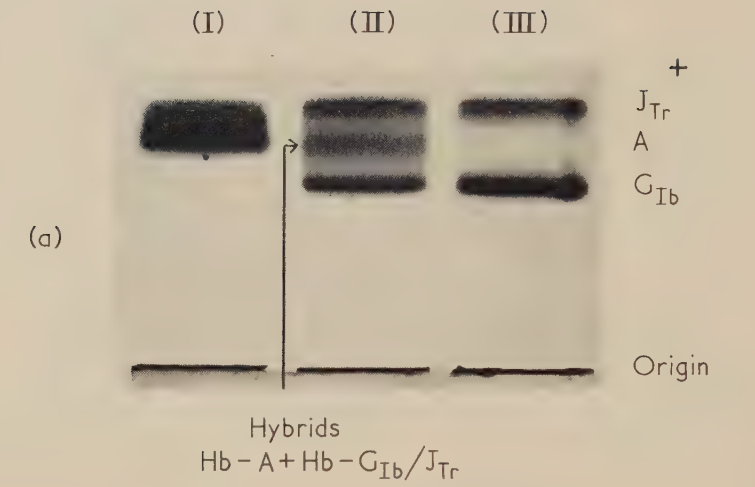


PLATE IV. Hybridization of haemoglobins J_{Tr} and S. Starch gel analyses in (a) discontinuous system, migration time 2 hr; benzidine stain, and (b) phosphate buffer, gel pH 7·4; migration time 2 hr, benzidine stain. (I) Hb-A, (II) untreated mixture of Hb-J_{Ib} and Hb-S (III) mixture of Hb-J_{Tr} and Hb-S dissociated and recombined.



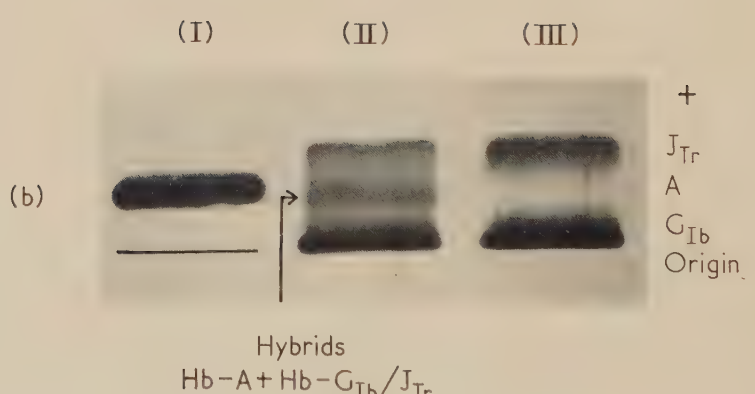


PLATE V. Hybridization of haemoglobins G_{Ib} and J_{Tr}. Starch gel analyses in (a) discontinuous system; migration time 2 hr; *o*-dianisidine stain, and (b) phosphate buffer, gel pH 7·4; migration time 2 hr; *o*-dianisidine stain. (I) Hb-A, (II) mixture of Hb-G_{Ib} and Hb-J_{Tr} dissociated and recombined, (III) untreated mixture of Hb-G_{Ib} and Hb-J_{Tr}.

For the detection of hybrid haemoglobins there would appear to be little to choose between the higher pH phosphate and the discontinuous systems (Plates IV and V), although the latter is to be preferred when the hybrid haemoglobin concentrations are less than those of the parent haemoglobins (Plate III).

The results of the recombination experiments of Hb-G_{Ib} with Hb-S and Hb-J_{Tr} are compatible with the assumption that Hb-G_{Ib} has abnormal α-chains compared with Hb-A and may be written $\alpha_2^{G_{Ib}}\beta_2^A$. With Hb-S the composition of the hybrids may be deduced from the following equation (the figures beneath each haemoglobin refer to the difference in net charge between this haemoglobin and Hb-A):

$$\underset{\text{Hb-}G_{Ib}}{2\alpha_2^{G_{Ib}}\beta_2^A} + \underset{\text{Hb-S}}{2\alpha_2^A\beta_2^S} \rightarrow \underset{\text{Hb-}G_{Ib}\text{/S}}{\alpha_2^{G_{Ib}}\beta_2^S} + \underset{\text{Hb-}G_{Ib}}{\alpha_2^{G_{Ib}}\beta_2^A} + \underset{\text{Hb-S}}{\alpha_2^A\beta_2^S} + \underset{\text{Hb-A}}{\alpha_2^A\beta_2^A}$$

$$\underset{+2}{\phantom{2\alpha_2^{G_{Ib}}\beta_2^A}} \quad \underset{+2}{} \quad \underset{+4}{\phantom{\alpha_2^{G_{Ib}}\beta_2^S}} \quad \underset{+2}{\phantom{\alpha_2^{G_{Ib}}\beta_2^A}} \quad \underset{+2}{} \quad \underset{0}{}$$

One hybrid is Hb-A whilst the other is a doubly abnormal haemoglobin, Hb-G_{Ib}/S, whose net charge relative to that of Hb-A is, like that of Hb-C, about + 4. New haemoglobin species migrating in the position of Hb-A and close to that of Hb-C were in fact observed in this hybridization experiment (Plate III). It would appear, since no new haemoglobins were formed when Hb-S was hybridized with Hb-J_{Tr}, that the latter has abnormal β-chains. Making this assumption the hybridization of haemoglobins G_{Ib} and J_{Tr} may be represented as follows:

$$\underset{\text{Hb-}G_{Ib}}{2\alpha_2^{G_{Ib}}\beta_2^A} + \underset{\text{Hb-}J_{Tr}}{2\alpha_2^A\beta_2^{J_{Tr}}} \rightarrow \underset{\text{Hb-}G_{Ib}}{\alpha_2^{G_{Ib}}\beta_2^A} + \underset{\text{Hb-}G_{Ib}\text{/}J_{Tr}}{\alpha_2^{G_{Ib}}\beta_2^{J_{Tr}}} + \underset{\text{Hb-A}}{\alpha_2^A\beta_2^A} + \underset{\text{Hb-}J_{Tr}}{\alpha_2^A\beta_2^{J_{Tr}}}$$

$$\underset{+2}{\phantom{2\alpha_2^{G_{Ib}}\beta_2^A}} \quad \underset{-2}{\phantom{2\alpha_2^A\beta_2^{J_{Tr}}}} \quad \underset{+2}{\phantom{\alpha_2^{G_{Ib}}\beta_2^A}} \quad \underset{0}{\phantom{\alpha_2^{G_{Ib}}\beta_2^{J_{Tr}}}} \quad \underset{0}{} \quad \underset{-2}{\phantom{\alpha_2^A\beta_2^{J_{Tr}}}}$$

In this case the doubly abnormal hybrid, Hb-G_{Ib}/J_{Tr}, has approximately the same net charge as Hb-A. In keeping with this scheme only one additional haemoglobin zone was found in this experiment and it had the mobility of Hb-A (Plate V). It is therefore clear that Hb-G_{Ib} has abnormal α-chains and is not identical with Hb-G_{Sa} which has abnormal β-chains.

Haemolysates from the individual with haemoglobin G disease do not contain the common minor haemoglobin Hb-A_2 but another minor haemoglobin which has been called Hb-G_2 (Shooter *et al.*, 1960). Hb-G_2 differs in charge from Hb-G_{Ib} by about the same amount as does Hb-A_2 from Hb-A. This means that the charge differences between Hb-G_2 and Hb-A_2 and between Hb-G_{Ib} and Hb-A are also similar, and it is therefore conceivable that Hb-G_2 is the naturally occurring hybrid of Hb-G_{Ib} and Hb-A_2, i.e. $\alpha_2^{G_{Ib}}\beta_2^{A_2}$. Such a hypothesis would require that Hb-A_2 differs from Hb-A only in the composition of the β-chains. Whilst recent reports on the amino acid differences between these two haemoglobins (Stretton & Ingram, 1960) partly support this idea, further experiments are indicated to resolve this point.

We are indebted to Dr. Robert L. Hill for discussions on haemoglobin nomenclature out of which arose the present scheme for distinguishing between the two specimens of haemoglobin G. We would also like to thank Dr. E. M. Crook for his interest in this work and for reviewing the manuscript and also Drs. J. P. Garlick and N. A. Barnicot for supplying the specimen of Hb-G_{Ib}. One of us (D. B. G.) wishes to acknowledge financial support from the Colonial Medical Research Committee and another of us (P. S. G.) from Grant H-4706 of the National Heart Institute.

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Mechanism of the Radiation-Induced Degradation of Nucleic Acids

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Aqueous solutions of DNA and of purines, pyrimidines, nucleosides and nucleotides have been irradiated with X-rays in the presence of oxygen and the nature of the chemical changes were investigated. Experiments have been carried out in which the relative reactivities of the free purine and pyrimidine bases were examined; it was found that these two classes of compounds show about equal reactivity towards the radiation-produced radicals. In the nucleosides and nucleotides the base components are the main site of chemical attack, reacting with 70 to 85% of the available radicals. In DNA, determinations of the extent of base destruction, including the yield of organic hydroperoxide, lead to an estimate of 80% for the extent of radical scavenging by the base components. This value is also in agreement with the observations that about 20% of the OH radicals attack the sugar moieties as deduced from the number of phosphate end-groups formed. It is suggested that hydrogen-bond breaking is the result of the permeation of water molecules into the double helix of the DNA at the site of a radiation-induced chain break. In 0·1% DNA solutions the number of hydrogen bond pairs broken is 6 to 7 per 100 ev; this gives a value of 14 to 15 hydrogen bond pairs broken per main chain scission. The nature of the hyperchromic effect in polynucleotides and the radiation induced viscosity changes in DNA solutions are briefly discussed.

1. Introduction

The occurrence of chemical changes in the irradiation of polynucleotides was established as long ago as 1949, when effects on the constituent bases as well as on the sugar moieties were noted (Scholes, Stein & Weiss, 1949; Scholes & Weiss, 1952). Such radiation-induced chemical reactions with the nucleic acids in aqueous systems are due to the formation of reactive species from the water molecules on absorption of ionizing radiations (Weiss, 1944, 1947, 1960). It was found in this early work that fragmentation of the polynucleotides was accompanied by an increase in the number of titratable acid groups, by liberation of inorganic phosphate and by the formation of small amounts of the free purine bases. From this it was concluded that glycosidic- and internucleotide-linkages were broken. Deamination and ring fission of the constituent bases was also observed. Mild hydrolysis of the irradiated solutions led to a further release of significant amounts of inorganic phosphate, from which it was concluded that labile phosphate esters were formed (Daniels, Scholes & Weiss, 1955). Irradiation of DNA solutions in the presence of oxygen was shown to lead to the formation of stable organic hydroxy-hydroperoxides on the pyrimidine base moieties of the nucleic acid molecule (Scholes, Weiss & Wheeler, 1956).

Scholes & Weiss (1953; 1954) also investigated the chemical effects of X-rays on some of the nucleic acid constituents, viz. purines, pyrimidines, ribo-nucleosides and -nucleotides. It was found that, similar to the nucleic acids, the bases were decomposed

with liberation of ammonia and ring fission; with larger doses of radiation some smaller fragments, e.g. urea, guanidine and oxalic acid, were also found. Chemical changes occurring on irradiation of e.g. adenosine and yeast adenylic acid include formation of ammonia, decarboxylation, increase in titratable acid groups and liberation of adenine. From the *nucleoside* some free ribose was produced, while the *nucleotide* was dephosphorylated but did not yield any free sugar or the corresponding nucleoside. Irradiation of uridine resulted in the formation of some free uracil and ribose. An overall mechanism covering the radiation-induced decomposition of these substances was put forward. Irradiation of purine and pyrimidine ribonucleotides in aqueous solution resulted also in the formation of labile phosphate esters which was traced to the radiation-induced formation of (activating) carbonyl groups in the pentose molecule. Hydroperoxides were shown to be formed from the pyrimidines and their nucleosides and nucleotides when irradiated in the presence of oxygen (Daniels, Scholes & Weiss, 1956).

Some further interesting effects of ionizing radiations on de-aerated aqueous solutions of guanosine and guanylic acid have recently been reported by Hems (1958), who found some destruction of the imidazole ring with the production of a formamido-pyrimidine compound.

To understand the exact nature of the radiation-induced chemical changes in nucleic acids more experiments on the behaviour of the components of DNA were required. In this paper, we present some of the results relating to purines, pyrimidines, nucleosides and nucleotides, as well as some recent data concerning certain radiation-induced chemical changes in DNA. All of the irradiations described below were carried out with X-rays (200 kv) in the presence of oxygen (1 atm) unless otherwise stated.

2. Pyrimidines, Purines, Nucleosides and Nucleotides

(a) *Pyrimidines*

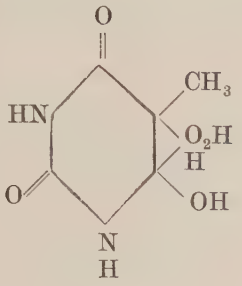
Aqueous solutions (2×10^{-4} M) of thymine, cytosine, uracil and dimethyl uracil were irradiated and the initial yields (G-values)† for the extents of destruction of the bases were determined. Three different methods were used: (i) spectrophotometry at an appropriate wavelength (measurements of this nature are only meaningful if none of the radiation products absorb at the particular wavelength used), (ii) paper chromatographic separation of the irradiated solutions, the individual pyrimidine spots being eluted and measured. In the case of thymine and also of uracil yet another check was possible by (iii) the determination of unchanged material by reaction with bromine.

The results are given in Table 1. Very good agreement was obtained using these various methods, particularly in the case of thymine. Cytosine was unusual in that it gave somewhat irreproducible results and this behaviour was traced to a post-irradiation effect, involving a slow decrease of the optical density of the irradiated solution. pH-Change from 1 to 7 had relatively little influence on the yield of thymine decomposed, whereas increase of the thymine concentration appreciably affected the extent of its radiolytic decomposition (Table 2).

The pyrimidines yield organic hydroperoxides on irradiation of their oxygenated aqueous solutions. In the case of thymine this was shown to be a hydroxy-hydroperoxide formed by the addition of one OH and one O_2H group to the 4 : 5 double

† The radiation yields have been expressed as G-values, i.e. the number of molecules changed per 100 ev of absorbed radiation energy.

bond, leading to the following structure (Weiss, 1958; Scholes & Weiss, 1960) (which still admits two stereoisomers).



This has been confirmed by chromatography and by comparing the rates of reaction with iodide ions of the radiation-produced hydroperoxide with those of the synthetic hydroxy-hydroperoxides (Weiss, 1958; Scholes & Weiss, 1960; Ekert & Monier, 1959).

TABLE 1

Initial yields of destruction of the pyrimidine bases in aqueous solution (2×10^{-4} M) *on irradiation with X-rays* (200 kv) *in the presence of oxygen* (1 *atm*), $pH = 5{\cdot}2$

Base	Yield (G)		
	u.v. method	Chromatography	Reaction with bromine
Thymine	1·89	1·91	1·89
Cytosine	2·05 to 2·28	2·16	—
Uracil	1·93	—	1·88
Dimethyl uracil	1·85	1·91	—

TABLE 2

Concentration dependence of the initial yields of the destruction of thymine on irradiation with X-rays (200 kv) *in the presence of oxygen* (1 *atm*), pH = 5·3

Thymine (mol. $l.^{-1}$)	Yield (G)
2×10^{-3}	2·76
2×10^{-4}	1·89
2×10^{-5}	1·23

The yields of hydroperoxide from several pyrimidines have been determined under various experimental conditions. Table 3 shows the influence of pH on the initial yields of thymine hydroperoxide and of hydrogen peroxide on irradiation of 2×10^{-4}

M-thymine solutions. Hydroperoxide formation depends markedly upon pH, the yield decreasing with decreasing pH. The effect of thymine concentration on the radiation yield is shown in Table 4. Cytosine hydroperoxide is unstable, particularly under neutral and alkaline conditions. The yields of hydroperoxide from neutral uracil solutions

TABLE 3

pH-Dependence of the initial yields of thymine hydroperoxide and hydrogen peroxide in aqueous solution (2×10^{-4} M) *on irradiation with X-rays* (200 kv) *in the presence of oxygen* (1 *atm*)

pH	G (Hydroperoxide)	G (H_2O_2)
8·00	1·02	1·81
5·08	1·05	1·73
3·05	0·58	1·79
1·95	0·33	2·35
1·09	0·14	2·69

(G = 1·16) were comparable to those obtained from thymine; dimethyl uracil also yielded a hydroperoxide in somewhat similar amounts (G = 0·75).

Comparison of the data for the yields of destruction of the pyrimidines and those of hydroperoxide formation show that whilst the latter compounds are the main products of irradiation, other substances must also be formed. It was observed that treatment of irradiated solutions of uracil and dimethyl uracil with sulphuric acid

TABLE 4

Dependence on thymine concentration of the initial yields of thymine hydroperoxide and hydrogen peroxide in aqueous solutions on irradiation with X-rays (200 kv) *in the presence of oxygen* (1 *atm*) pH = 5·3

Thymine (mol. $l.^{-1}$)	G(Hydroperoxide)	G(H_2O_2)
$3·2\times10^{-2}$	1·73	1·86
$3·2\times10^{-3}$	1·31	1·71
$3·2\times10^{-4}$	1·12	1·73
$3·2\times10^{-5}$	0·71	1·78

led to an increase in absorption at 260 mμ. The products responsible for this increased absorption were still present after the hydroperoxide had been removed. In the case of dimethyl uracil the nature of the product could be studied more easily, since unchanged pyrimidine could be removed from the irradiated solution by extraction with chloroform. The spectrum in acid solutions of the separated radiation product agreed well with that obtained on acid treatment of solutions of dimethyl uracil glycol; it was also found that such treatment of both the radiation product and di-

methyl uracil glycol gave dimethyl iso-barbituric acid. Hence irradiation of dimethyl uracil solutions in the presence of oxygen leads to the formation of dimethyl uracil glycol. The initial yield in 2×10^{-4} M-dimethyl uracil solutions at pH 5·5 was found to be G(glycol) = 0·4; in acid solution (pH $\sim$ 2) no glycol appeared to be formed.

In the case of uracil, glycol is also an irradiation product, corresponding to a yield of G(glycol) = 0·45 at pH 5·5. As with dimethyl uracil, no glycol was detected in acid solutions.

Irradiated solutions of thymine and cytosine did not show any increase in absorption at 260 mμ on acid treatment. However, these findings do not necessarily indicate the absence of glycol since it has been found that acid treatment of solutions of synthetic thymine glycol does not lead to spectral changes.

No lower aldehydes or dialdehydes could be detected. It appears, therefore, that the main products of irradiation of pyrimidines are those formed by saturation of the 4 : 5 double bond and that no ring-opening occurs.

In this paper the decomposition of water by ionizing radiations is represented by equation (1), leading to the free radicals H and OH. However, it is very likely that ionic species are also formed (Weiss, 1944; 1947) and that these can enter into chemical reactions with the solute. The exact nature of the species does not affect the discussion of the stoicheiometry and at this stage, for the sake of simplicity, we have designated the reactive entities as H and OH.

The quantitative data on the extent of degradation of the pyrimidines can be accounted for if we consider that the OH radicals (but not HO_2) react with the solute in solutions irradiated in the presence of oxygen. For X-rays (200 kv) the OH radical yield should be G(OH) 2·0 to 3·0 depending upon solute concentration. In fact, the stoicheiometry in neutral oxygenated systems can be accounted for by the following reaction scheme (A = pyrimidine):

$$H_2O \text{ —}\sim\!\!\sim\!\!\rightarrow H,\ OH,\ H_2,\ H_2O_2 \qquad (1)$$

$$A + OH \rightarrow A(OH)\cdot \qquad (2)$$

$$A(OH)\cdot + O_2 \rightarrow A(OH)O_2\cdot \qquad (3)$$

$$H + O_2 \rightarrow HO_2 \rightleftharpoons H^+ + O_2^- \qquad (4)$$

$$A(OH)O_2\cdot + O_2^- \rightarrow A(OH)O_2^- + O_2 \qquad (5)$$

$$2A(OH)O_2\cdot \rightarrow \text{glycol } (+ \text{ other products}) \qquad (6)$$

$$2HO_2 \rightarrow H_2O_2 + O_2 \qquad (7)$$

In this mechanism reaction (4) and (5) are the pH-dependent steps and would imply a decrease in hydroperoxide yield with increasing acidity. In neutral solution the yield of hydrogen peroxide as a function of thymine concentration (Table 4) can be accounted for if, at the lower concentrations, a back reaction with hydrogen peroxide takes place, viz.

$$OH + H_2O_2 \rightarrow H_2O + HO_2 \qquad (8)$$

(b) *Purines*

Table 5 gives values for the extent of destruction of adenine, guanine, xanthine and uric acid. Fairly good agreement was obtained using the three methods, viz. (i) decrease of optical density at an appropriate wavelength, (ii) chromatographic separation, and (iii) precipitation of unchanged purine as the silver salt. In the case of adenine, careful analysis failed to reveal the presence of any significant amounts of radiation products absorbing in the region of 263 mμ. A similar result has also been

found by Hems (private communication). In some experiments very small amounts of the oxyadenines appeared to be present but they were formed with yields of less than G $\sim$ 0·1. Similarly very small amounts of uric acid were formed from xanthine. The concentration dependence of the radiation-induced decomposition of adenine is shown in Table 6.

TABLE 5

Yields of destruction of purines in aqueous solutions (2×10^{-4} M) *on irradiation with X-rays* (200 kv) *in the presence of oxygen* (1 *atm*), pH = 5·1

Purine	Yield (G(−base))		
	u.v. method	Chromatography	Precipitation as silver salt
Adenine	1·09	1·13	1·00
Xanthine	1·36	1·32	1·40
Uric acid	1·39	1·48	1·31

We have previously reported that purines do not form hydroperoxides on irradiation in oxygenated aqueous systems (Scholes *et al.*, 1956). The chemical changes that occur lead to the liberation of ammonia and with larger doses of radiation, ring opening and fragmentation to smaller molecular units occurs. However, we know

TABLE 6

Concentration dependence of the initial yields of adenine destruction in aqueous solutions on irradiation with X-rays (200 kv) *in the presence of oxygen* (1 *atm*), pH = 5·2

Adenine (mol. l^{-1})	Yields (G(−adenine))
2×10^{-3}	1·10
2×10^{-4}	1·09
2×10^{-5}	0·65

relatively little about the detailed mechanism of the radical attack in the purine molecule, although it has been postulated that the OH radicals attack the central ethylenic double bond (Scholes & Weiss, 1953, 1954).

In neutral solutions, there is a striking difference in the extent of destruction of the purines and pyrimidines; the low yield for adenine is particularly noticeable. However, at pH 1·2, the yield for the destruction of adenine was found to be G = 1·9, a value which is very similar to that for the pyrimidines under similar conditions. The low yields in neutral solution could be explained if (a) purines are intrinsically less reactive towards the free radicals, (b) there is multiple radical attack, and (c) there is a subsequent reaction step which can cause "restitution" to the original purine molecule.

Experiments were carried out in which the relative reactivities of the purine and pyrimidine bases were examined.

Solutions of mixtures of various purines and pyrimidines were irradiated and the extents of radiolytic decomposition of the individual components determined by

TABLE 7

Yields for the competitive destruction of purine and pyrimidine bases and of hydroperoxide formation on irradiation in aqueous solutions with X-rays (200 kv) *in the presence of oxygen* (1 *atm*), pH = 5·3

(a) Adenine-thymine

Solution	Yields		
	G(−adenine)	G(−thymine)	G(hydroperoxide)
10^{-4} M-adenine–10^{-5} M-thymine	1·05	0·23	—
10^{-4} M-adenine–10^{-4} M-thymine	0·58	1·02	—
10^{-5} M-adenine–10^{-4} M-thymine	0·21	1·41	—
10^{-3} M-adenine–10^{-3} M-thymine	—	—	0·64
10^{-3} M-adenine–10^{-4} M-thymine	—	—	0·13

(b) Thymine-cytosine

Solution	Yields		
	G(−thymine)	G(−cytosine)	G(hydroperoxide)
5×10^{-4} M-thymine 5×10^{-4} M-cytosine	0·92	1·02	0·77

(c) Thymine, cytosine, adenine, guanine

Solution	Yields				
	G(−thymine)	G(−cytosine)	G(−adenine)	G−(guanine)	G(hydroperoxide)
Each base at a concentration of $3{\cdot}3 \times 10^{-5}$ M	0·40	0·38	0·22	0·17	0·28

paper chromatographic separation. Table 7 shows some results obtained. From these experiments one must conclude that the purines and pyrimidines examined show about equal reactivity towards the radiation-produced radicals. The possibility of multiple radical attack in the purines is rather unlikely on general grounds, and

particularly also in view of the similar concentration dependence of thymine and adenine (Table 4 and 6). At this stage, therefore, one can only surmise that in neutral solutions there is a back reaction leading to the re-formation of the purine. From the above experiments one would deduce that, whereas one OH radical can destroy one molecule of thymine, cytosine or uracil, the consumption of approximately two OH radicals is necessary for the destruction of one molecule of adenine or guanine.

(c) *Nucleosides and nucleotides*

Certain features of the radiation-induced chemical changes in nucleosides and nucleotides have been discussed previously (Scholes, 1953; 1954; Daniels *et al.*, 1956). In this paper we want to present some quantitative data on the extent of destruction of the base moieties in these molecules, in so far as this leads to an evaluation of the relative reactivities. These experiments were again carried out in the presence of oxygen and the extent of base destruction was measured spectrophotometrically. Table 8 records some of the values obtained. Some results for the free bases are

Table 8

Yields of destruction of base components of various purine and pyrimidine nucleosides and nucleotides on irradiation of their aqueous solutions (2×10^{-4} M) *with X-rays* (200 kv) *in the presence of oxygen* (1 *atm*)

Compound	Yield (G(− base))
Uracil (pH 5·3)	1·95
Uridine (pH 5·3 to 7·0)	1·72
Uridylic acid (pH 3·8 to 7·0)	1·65
Thymine (pH 7)	1·90
Thymidine (pH 7)	1·75
Thymidylic acid (pH 7)	1·65
Adenine (pH 7)	1·09
Adenosine (pH 7)	0·92
Adenylic acid (3′) (pH 7)	0·75

included for comparison. In the compounds investigated the extent of base destruction decreases in the order: free base, nucleoside, nucleotide. However, the presence of the ribose or ribophosphate residues does not drastically change the extent of chemical attack on the base. In adenylic acid, about 30% of the radicals react with the pentose molecule whereas in uridylic acid only about 15% are thus diverted. Ion-exchange chromatography of irradiated solutions of adenylic acid showed that some ultraviolet absorbing products other than free adenine are produced. We have confirmed the findings of Hems that the spectral changes associated with the formation of a pyrimidine formamido-compound in irradiated solutions of guanylic acid are not found in solutions irradiated in the presence of oxygen. If the opening of the imidazole ring is due to OH radicals then the same site should be attacked in the presence of oxygen and under these circumstances the fact that the same end product is not formed in oxygenated solutions would not be surprising in view of the fact that oxygen has a profound effect on the subsequent reactions involving the primary organic radical (Scholes & Weiss, 1959).

3. Nucleic Acids

(a) *Formation of hydroperoxides*

Further experiments have been carried out on the yields of the radiation-produced hydroperoxide in DNA. Solutions of different concentrations of DNA were irradiated with X-rays (200 kv) in the presence of oxygen and the amounts of hydroperoxide determined, after removal of the hydrogen peroxide with catalase (Table 9). The most

TABLE 9

Concentration dependence of the initial yields of DNA hydroperoxide in aqueous solutions irradiated with X-rays (200kv) *in the presence of oxygen* (1 *atm*)

DNA (wt. %)	G(hydroperoxide)
0·005	0·17
0·025	0·30
0·10	0·55

dilute DNA solutions used (0·005%) correspond to a total concentration of bases of about 10^{-4}M. The yield of hydroperoxides obtained by irradiation of a mixture of the bases (thymine, cytosine, adenine and guanine) of comparable concentration was found to be G(hydroperoxide) = 0·22, which shows that the extent of hydroperoxide formation is reduced only about 20% by the presence of deoxyribophosphate residues. Experiments with deoxyribonucleotides have shown that the deoxyribose component does not form a hydroperoxide and that in neutral solutions only thymidylic acid forms a stable hydroperoxide. We conclude, therefore, that the stable hydroperoxide found in irradiated DNA solutions is essentially associated with the thymine molecule. It also appears that the thymine hydroperoxide remains attached to the DNA since it is completely precipitated on treatment of the irradiated solution with trichloroacetic acid.

(b) *Destruction of purine and pyrimidine bases*

Irradiated DNA solutions were frozen dried and hydrolysed with formic acid or with perchloric acid. The liberated bases were separated by paper chromatography and the yields of destruction (G-values) of the individual bases were obtained by u.v. absorption of the eluates. Fairly reproducible results were obtained with a given sample (Table 10).

The total number of OH radicals attacking the bases of DNA can be placed between two limiting values, assuming either 100% or 50% efficiency of the reactions with the purines. In the case of 0·005% DNA solutions these limits are G = 0·99 and G = 1·42. From a synthetic mixture of the bases at comparable concentrations (Table 7) the total yield of OH radicals reacting with the components comes to G = 1·56, taking into account that under these conditions there is 50% efficiency in the adenine and guanine reactions. In DNA, therefore, a minimum of 63% and a maximum of 90% of the available radicals react with the bases. These limits agree very well with those derived from measurements on the nucleotides (Table 8). A value of 80% of OH

radicals reacting was arrived at by measurements of the yields of hydroperoxide (see above). Until more accurate data are available this should be taken as a fair estimate of the extent of radical scavenging by the bases. By direct measurement of the change in u.v. absorption at 260 mμ of irradiated DNA solutions, Barron, Johnson & Cobure (1954) concluded that attack on DNA was about one to four thousand times less than that on the free bases at comparable concentrations. This observation

TABLE 10

Initial yields of destruction of purine and pyrimidine bases on irradiation of aqueous solutions of DNA with X-rays (200 kv) *in the presence of oxygen* (1 *atm*)

DNA concentration (wt. %)	G(− base)			
	Thymine	Cytosine	Adenine	Guanine
0·005	0·33	0·23	0·25	0·18
0·200	0·64	0·38	0·39	0·26

has been taken by Alexander & Stacey (1958) to indicate that the sugar-phosphate component has a much greater reactivity for the free radicals than the purines and pyrimidines. However, such measurements are not meaningful since it is known that the simultaneous breaking of hydrogen bonds leads to an increased u.v. absorption (hyperchromic effect) and thus the experimentally observed optical densities are the net balance of this increase and the decrease due to destruction of the bases.

(c) *Chemical attack on the sugar phosphate components*

It appeared possible to measure directly the number of breaks in the sugar-phosphate backbone by treating the irradiated solutions with prostate phosphomonoesterase which liberates inorganic phosphate from phosphate end-groups, since at the site of each break a phosphate end-group will be formed. Experiments with this enzyme have shown that such end-groups are in fact formed (B. Collyns & S. Okada, unpublished results). In irradiated 0·1% DNA solutions, for example, it has been found that the number of phosphate end-groups corresponds to a yield of G ~ 0·4 to 0·5. The yield of base destruction at this concentration is of the order G ~ 2·0. These results are thus substantially in agreement with the above conclusion that about 80% of the radicals react with the bases and about 20% with the sugar moieties. This also shows that essentially every attack on the deoxypentose leads to breakage of the internucleotide ester linkage. The yield of the breakage of the phosphodiester linkage can also be calculated under certain assumptions from physical measurements (Peacocke & Preston, 1959): this leads to a value of G = 2·3, which is much higher than that obtained by the direct phosphate end-group determinations. Although it has not been proved that the value derived from the latter method is entirely reliable, the lower value agrees much better with the overall chemical attack on the DNA and is likely to be much nearer to the true value than the calculated one.

(d) *Breakage of hydrogen bonds*

Cox, Overend, Peacocke & Wilson (1955) and Peacocke & Preston (1959) have shown by titration studies and by ultraviolet absorption measurements that hydrogen bond rupture is an important consequence of the chemical action of ionizing radiations on DNA in aqueous solution.

Examination of the change in ultraviolet absorption of irradiated DNA solutions leads to an estimate of the extent of hydrogen bond breakage (Collyns & Okada, unpublished results). DNA solutions irradiated with different doses were divided into two parts, one being adjusted to pH 3·2 and the other to pH 7·7 and the optical densities at 260 mμ measured. It was found that the optical density of irradiated DNA solution at pH 3·2 decreased linearly with radiation dose due to the destruction of the chromophores (base moieties). At pH 7·8, on the other hand, the optical density first increased (hyperchromic effect) due to breakage of hydrogen bonds and then decreased, due to increasing effects of base destruction. From these results the approximate initial yields of hydrogen bond breakage can be estimated. It was found that in 0·1% DNA solutions 6 to 7 hydrogen bond pairs are broken per 100 ev energy absorbed. Although chemical destruction of the bases may contribute to this process this would be too small to account for the observed large changes. Taking the number of end-groups formed at G $\sim$ 0·45, the number of hydrogen bond pairs broken per chain break amounts to a yield of about G $\sim$ 14·0 or 15·0; this agrees fairly well with the value of 16·0 obtained by Peacocke & Preston (1959).

The fact that a relatively large number of hydrogen bond pairs can be broken per single chain break can be explained if one assumes that, following the chain break, water molecules can permeate into the double helix of the DNA. In this way hydrogen bonding between the conjugate base pairs will be replaced to some extent by hydrogen bonding between the individual bases and the water molecules: the "unzipping" of the polynucleotide strands in this way must be a limited process.

It is of interest that on the basis of certain theoretical considerations one arrives also at a similar figure for the number of hydrogen bonds "unzipped". In a system where two chains are twisted around one another, the entropy gain per unwound link at first increases because a link with one end free can range through a greater volume in space. As more and more of the chain becomes unwound, the number of possible configurations becomes restricted. Thus the entropy gain per unwound link increases, passes through a maximum and finally diminishes to some limiting value as the length of the unwound chain has increased beyond a certain value. There will, therefore, be a tendency to get an "unzipping" effect of several links until an "equilibrium length" is reached. In the case of DNA, this untwisting is due to the rupture of hydrogen bonds and it has been calculated by Temperley (1959) that the untwisted length is of the order of 15 to 20 links.

(e) *The hyperchromic effect*

Considerable attention has been directed towards the so-called "hyperchromic effect" in view of its possible importance for the elucidation of the structure of nucleic acids. A commonly accepted explanation of this effect is, that it is due to some interaction between the π-electrons of the different bases. However, it should be borne in mind that the distance between adjacent base molecules in DNA is of the order of 3 to 4 Å and it is, therefore, most unlikely that any appreciable electronic interaction (overlap) can occur at these relatively large distances.

It is suggested that there is an essentially more straightforward explanation for this hyperchromic effect along the following lines.

The absorption spectra of the purine and pyrimidine bases will, in conformity with those of other polar molecules, be dependent upon the polarity of the surrounding medium. Light absorption by these bases is governed by the system of conjugated double bonds and, in an aqueous solution, more particularly by the hydrogen bonding between the polar groups of the base with the solvent, water. If one considers an intact DNA molecule with its double helical structure (Watson-Crick model), hydrogen bonding is operative between the appropriate pair of bases opposite to each other. Thus, in DNA each molecule of base is present in the "medium" of other purine or pyrimidine molecules and its absorption spectrum will be largely influenced by this environment. If, however, the two polynucleotide strands become separated, e.g. by the interpenetration of water, hydrogen bonding between the bases in the two strands will be replaced, to a greater or lesser extent, by hydrogen bonding between the base molecules and the water. This, therefore, would suggest that the hyperchromic effect is due to a change from hydrogen bonding between the bases in the intact helix to hydrogen bonding of the base moieties with the surrounding water.

(f) *Viscosity changes and "after-effect"*

The detailed chemical studies of the radiation-induced changes in purines, pyrimidines, nucleosides and nucleotides as outlined above, have led to important conclusions regarding the points of attack by the radiation-produced free radicals and the nature of the compounds thus formed. Experiments with DNA have substantiated the fact that many of these conclusions apply to the polynucleotide itself. That the bases are within the helix and surrounded by sugar-phosphate does not appreciably affect the relative reactivity of these components towards the free radicals. The predominant attack of the radicals is on the bases of the polynucleotide.

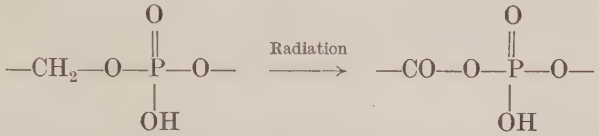
Unfortunately, there are no available data which give unambiguous results concerning molecular weight changes in the early stages of radiation damage, or even more significantly, of molecular weight changes throughout the period of the after-effect. The conclusions of Cox *et al.* (1955) that the number of scissions of the DNA molecule is proportional to the square of the radiation dose were derived from experiments in which extensive degradation of the polynucleotide had taken place. If a radiation-induced internucleotide break occurs within about 20 nucleotide units of another break (which may itself be radiation-induced or may be a natural break) then as a result of unzipping of the type discussed above, smaller molecular single chain fragments could be split off from the main DNA particle.

Although the viscosity of DNA solutions may be markedly reduced on irradiation this does not necessarily imply changes in molecular weight; as Eisenberg (1957) has pointed out, molecular weight is not decisive even for a qualitative characterization of the viscosity behaviour of DNA solutions. It is very likely that part of the loss in viscosity must be attributed to a change of the molecular configuration as a result of chemical damage in the backbone of one of the strands of the polynucleotide chain. Main chain breakage of one of the strands would lead to a greater degree of flexibility of the double helical structure which would then lead to a *bending over* or *coiling* at the site of the break and should thus result in a change in the viscosity of the DNA solutions. This would be in agreement with the DNA model proposed by Eisenberg (Gaussian coil with high internal viscosity).

The theory of the viscosity of polyelectrolytes is very complex and great caution

must be exercised in interpreting viscosity data and particularly the viscosity "after-effect." As yet, there is no satisfactory chemical explanation for this effect, but if we assume that this phenomenon is associated with main chain breakage then two possibilities may be suggested:

(1) acyl-phosphates are formed on the sugar entities, viz.



These are known to be formed from simple phosphate mono- and diesters in solutions irradiated in the presence of oxygen (Scholes & Weiss, 1954). They are labile towards hydrolysis and could thus account for post-irradiation internucleotide breakage. Unfortunately, with the rather crude analytical methods available, it has not been possible to establish with any certainty the presence of acyl compounds in irradiated DNA solutions.

(2) organic hydroperoxides which are formed on the pyrimidine bases. The experimental evidence suggests that any after-effect from this source could arise from some effects on hydrogen bonding or internucleotide links as a result of possible fission of the pyrimidine glycosidic linkage. The observed rates of DNA hydroperoxide decay are of the same order, although not identical with the rate of post-irradiation viscosity loss (Daniels, Scholes, Weiss & Wheeler, 1957). It is, therefore, not impossible that there may be some connection between these two phenomena.

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The Transformation of *Escherichia coli* with Deoxyribonucleic Acid isolated from Bacteriophage *λdg*†

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Deoxyribonucleic acid of *λdg*, isolated by phenol extraction, will transform galactose-negative strains of *Escherichia coli* K12. That the transforming agent is indeed DNA is shown by (1) its sensitivity to pancreatic DNase, (2) its resistance to anti-*λ* antibody, (3) its resistance to heat up to the characteristic melting temperature of *λdg* DNA, and (4) its buoyant density of 1·71 g cm^{-3}.

Transformation is found to occur only if the bacteria exposed to *λdg* DNA are simultaneously infected with ordinary *λ*.

The transforming agent appears to be the entire *λdg* chromosome since the phage genes *c* and *mi* are also present in the galactose-positive transformants.

1. Introduction

This paper describes a system for the genetic transformation of *Escherichia coli* K12. Recipient strains which are unable to metabolize galactose because they lack the enzymes galactokinase or galactose-1-phosphate uridyl transferase are transformed into galactose-metabolizing strains. The transforming agent is DNA isolated from *λdg*, a variant of coliphage *λ*.

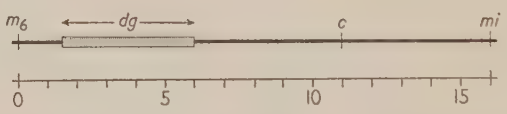

Fig. 1. This linkage map shows the position of the "*dg* region" relative to the phage genes m_6, *c*, and *mi*. The *dg* region is defined as that part of the *λ* chromosome which is not recoverable in active phage when *λdg* is crossed to normal *λ*. Data for the size and position of *dg* were taken from the experiments of Arber (1958). The distances are measured in recombination units whereby a cross involving two markers separated by one recombination unit gives 1% recombinants.

Lambda is a temperate phage whose locus on the chromosome of *E. coli* K12 is closely linked to the genes controlling several enzymes of galactose metabolism (Lederberg & Lederberg, 1953). Ultraviolet irradiation of bacteria lysogenic for *λ* initiates synthesis and release of phage particles. Most of the new phage particles are ordinary *λ*. However, approximately one in 10^6 of the new particles is *λdg*.

Lambda *dg* transduces galactose genes from the bacterium in which it was produced to the bacterium it infects (Morse, Lederberg & Lederberg, 1956). The transduced galactose genes control at least the enzymes galactokinase and galactose-1-phosphate uridyl transferase (Kalckar, Kurahashi & Jordan, 1959). The acquisition of galactose

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genes by *λdg* is accompanied by the loss of a part of the genetic material present in ordinary λ. The region which is inactive or absent in *λdg*, i.e., which is defective, is indicated on a linkage map of λ given in Fig. 1.

The defectiveness of *λdg* is expressed in the following way. Of the bacteria infected with ordinary λ under standard conditions, 20% become lysogenic. Under the same conditions only 1% of the bacteria infected with *λdg* become lysogenic. However, if bacteria are infected simultaneously with *λdg* and λ, then 20% become lysogenic for both *λdg* and λ (Arber, 1958). Thus ordinary λ acts as a "helper" for the lysogenization of *λdg*, presumably by supplying a normal *dg* region.

The defectiveness of *λdg* is expressed in another way. Bacteria lysogenic for *λdg*, induced with ultraviolet light (u.v.), lyse but do not produce any phage (Arber, 1958). However, doubly lysogenic bacteria, carrying both *λdg* and λ, induced with u.v., lyse and produce both *λdg* and λ. Lambda *dg* can be obtained substantially free of ordinary λ by density gradient centrifugation because *λdg* differs slightly in buoyant density from λ (Weigle, Meselson & Paigen, 1960).

Lambda *dg* would seem to be a good potential source of genetically active DNA. The DNA complement of one *λdg* particle is about 1/100 that of an *E. coli* cell, and since both carry the same galactose genes, the fraction of the DNA represented by the genes for galactokinase and galactose phosphate uridyl transferase is about one hundred-fold higher in *λdg* DNA than in *E. coli* DNA. Moreover, during the purification of *λdg*, the protein coat protects its charge of DNA from destruction by nucleases released during cell lysis. Finally, the knowledge of the genetic structure of λ (Jacob & Wollman, 1954; Kaiser, 1955) and *E. coli* K12 (Lederberg, Lederberg, Zinder & Lively, 1951; Wollman, Jacob & Hayes, 1956) provides a basis for the interpretation of experiments with isolated DNA.

DNA isolated from *λdg* is shown in this paper to be active in transformation. Phenol extraction of *λdg* denatures its protein and releases its DNA into aqueous solution. The *λdg* DNA, so prepared, will transform strains of *E. coli* K12 which lack either galactokinase or galactose transferase. Transformation is found to occur, however, only if the bacteria exposed to *λdg* DNA are simultaneously infected with "helper" phage (e.g. ordinary λ). Analysis of the transformed bacteria shows that the active DNA carries phage genes as well as galactose genes, and, in fact, appears to be the entire chromosome of *λdg*.

2. Materials and Methods

(a) *Media*

The growth media used include: Difco bacto-tryptone broth with 0·5% NaCl (TB medium) and its agar derivatives, TB soft agar (0·7% agar) and TB plate agar (1% agar); EMB-galactose agar as described by Lederberg (1950) except that 10 g/l. Difco bacto-tryptone is substituted for 8 g/l. of casein digest; H medium consisting of 0·1 M-potassium phosphate buffer, pH 7·0, 0·015 M-$(NH_4)_2SO_4$, 0·001 M-$MgSO_4$ and $1{\cdot}8 \times 10^{-6}$ M-$FeSO_4$; and P medium which is identical to H except that the phosphate concentration is 0·02 M.

Unless otherwise specified, bacteriophage stocks were kept in and diluted into a solution (termed λ-dil.) of 0·01 M-potassium phosphate buffer, pH 7·0, 0·01 M-$MgSO_4$, and 10 μg/ml. of bovine plasma albumin.

(b) *Special reagents*

The trypsin and pancreatic DNase were crystalline products of Worthington Biochemical Corp. Thymus DNA was prepared according to the procedure of Kay, Simmons & Dounce (1952). CsCl was obtained from American Potash and Chemical Corp.; its analysis

in per cent by weight was: CsCl—95·01; RbCl—4·11; KCl—0·39; NaCl—0·06; $NaHCO_3$—0·02; and moisture—0·03. The CsCl concentrations referred to in the text are not corrected for the presence of these impurities. For experiments involving the centrifugal banding of DNA in a CsCl gradient, ultraviolet light-absorbing impurities in the CsCl were reduced by heating the CsCl at 500°C for about 20 hr and then passing a 60% solution of the heated CsCl through a Norite column. The resulting solutions had absorbancies of less than 0·05 at 260 mμ. All other chemicals were C.P. grade.

(c) *Bacterial strains*

The galactose-negative mutants of *E. coli* K12 were isolated by Dr. E. Lederberg who generously made them available to us. Mutant W3102 (Gal_2^-) is defective in the synthesis of galactokinase, mutants W3101 (Gal_1^-) and W3104 (Gal_4^-) are defective in the synthesis of galactose-1-phosphate uridyl transferase. All three strains are nonlysogenic. The three were derived from W3092, W3091, and W3094, respectively, whose enzyme defects have been analyzed (Kalckar *et al.*, 1959). The double mutant $Gal_1^-Gal_4^-$ was isolated by Dr. M. L. Morse (stock no. 550) who kindly permitted us to use it. It is also methionine-requiring, lactose-negative, T1-resistant, streptomycin-resistant, and F^-.

Strain C600 is a nonlysogenic, galactose-positive derivative of K12 (Appleyard, 1954).

Lysogenic derivatives of these strains were isolated from the survivors of phage infection. The symbol for a lysogenic strain, e.g. C600(λ), is read: C600 lysogenic for λ.

(d) *Bacteriophages*

Wild type λ, implied whenever the symbol λ is written without qualification, forms turbid plaques about 3 mm in diameter. Its origin has been described (Kaiser, 1957).

The plaques formed by λ *co mi* are clear (*co*) and minute, with well defined halos (*mi*). The *co* mutation, which is very closely linked to *c*, gives 5% recombination with *mi* (Fig. 1) (Kaiser, 1957).

λi^{434}, which has also been called 434 *hy*, is identical with λ except that it has the immunity specificity gene of 434. The i^{434} and i^{λ} genes are two different alleles at the *c* locus (Kaiser & Jacob, 1957).

Isolation of λdg. A stock containing 10^9 λ and 10^2 λdg/ml. was obtained by u.v. irradiation of the galactose-positive strain C600(λ). It is known that several types of λdg, differing in buoyant density, occur in such a stock (Weigle *et al.*, 1960). All of the experiments reported here involve the same λdg, which was isolated as follows. K12 Gal_4^- was exposed to the aforementioned stock and a single galactose-positive colony isolated. Cultures grown from this colony, when induced with ultraviolet light, produce both λ and λdg. It is, therefore, lysogenic both for λ and λdg, symbolized Gal_4^- (λ, λdg). To facilitate the preparation of u.v.-induced lysates from Gal_4^- (λ, λdg), a mutant unable to adsorb λ was selected from it: Gal_4^- (λ, λdg)/λ. This strain served as the source of λdg.

Concentrated stocks of λ, λi^{434}, and λ *co mi* to be used as helper were prepared by u.v. induction of Gal_4^- (λ), Gal_4^-(λi^{434}), and Gal_4^- (λ *co mi*), respectively, and then purified by the procedure described in a succeeding section for λdg.

(e) *Assays*

λ.—The number of active λ in a phage suspension was determined by counting the number of plaques produced by an appropriately diluted sample of the suspension using the agar layer technique described by Adams (1959). The indicator bacteria were strain W3104.

λdg.—The number of functional λdg particles in a phage suspension was determined from the number of galactose-positive colonies produced by an appropriately diluted sample in a standard transduction assay. The assay was carried out as follows: an 0·1 ml. portion of the λdg to be assayed was mixed with 0·2 ml. of exponentially growing cells of W3104 in TB medium (2×10^9 cells/ml.) and 0·1 ml. of helper phage λ (8×10^9 λ/ml.). The mixture was incubated at 37°C for 20 min, then 2 ml. of soft TB agar was added and the entire mixture poured onto an EMB-galactose plate. After incubation for 40 to 48 hr at 37°C, the plates were scored for Gal^+ colonies.

Since 20% of the λdg lysogenize under these conditions, the number of λdg particles is obtained by multiplying the number of Gal^+ colonies by 5. The validity of this procedure

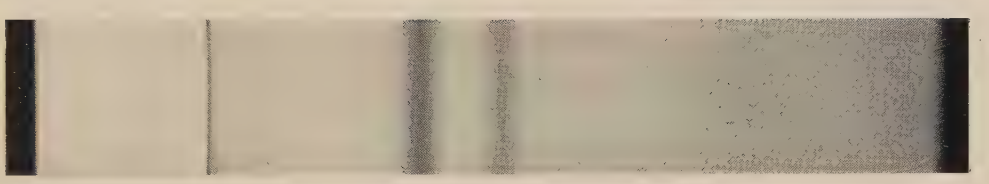

PLATE I. The separation of *λdg* and *λ* phage in a cesium chloride gradient after 8 hr centrifugation at 34,410 rev/min, 5°C. This phage suspension was similar to fraction CsCl-1 except that $\rho_4 = 1{\cdot}51$. The more concentrated band of ultraviolet-absorbing material nearest the meniscus is *λdg* and has a buoyant density of 1·49. The other band is *λ* with a density of 1·50. These buoyant densities are mean values determined by direct density measurements of the fractions obtained during the preparation of fraction CsCl-3 ("Materials and Methods").

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is confirmed by the observation that in purified preparations of λ the ratio of active λ to absorbancy at 260 mμ is 5 times larger than the ratio of *Gal*$^+$ to absorbancy at 260 mμ for purified *λdg*.

(f) *Chemical and physical determinations*

Orthophosphate was estimated by the method of Fiske & SubbaRow (1925) and total phosphorus was measured as orthophosphate after digestion in concentrated H_2SO_4 to which H_2O_2 was added. The diphenylamine reaction of Dische (1955) was used to determine deoxypentose. Pentose was determined by the Mejbaum (1939) procedure, using a 40-min heating period and adenosine 5′-phosphate as a standard. Protein was determined by the phenol method of Lowry, Rosebrough, Farr & Randall (1951) using crystalline bovine plasma albumin as a standard. Absorbancy at a single wavelength was determined in a Beckman model DU spectrophotometer using a 1·00 cm light path, whereas absorption spectra were measured in a Cary model 14 recording spectrophotometer. The viscosity of DNA solutions was determined with a capillary viscometer (Schachman, 1957) at concentrations of about 25 μg/ml. Densities of cesium chloride solutions were determined with a 0·2 ml. pycnometer.

(g) *Purification of λdg*

A culture of Gal_1^- (λ. *λdg*)/λ growing exponentially in H medium containing 1% galactose was induced by irradiation with u.v. after it had achieved a concentration of 2×10^9 cells/ml. and had been cooled to 0°C. After irradiation of 50 liters of culture, 12 liters of 10% Difco bacto-tryptone broth were added, the mixture brought to 37°C, and incubated with aeration until maximum lysis occurred (measured by the absorbancy of the culture at 600 mμ). This lysate was immediately cooled to 0°C and centrifuged in a Sharples centrifuge to clear it of bacterial cells and debris. The supernatant fraction is termed the *crude lysate*. All further purification steps were performed in the cold room at 4 ± 1°C.

The crude lysate was concentrated by adding 250 g of $(NH_4)_2SO_4$/liter of lysate and allowing a precipitate to form and settle to the bottom of the container by letting the mixture stand overnight. After siphoning off the clear supernatant fluid, the precipitate-containing portion was centrifuged to yield a pellet containing the phage. This pellet was taken up in λ-dil. to a final volume one-fortieth of that of the crude lysate and, after dialysis against 0·01 M-$MgSO_4$ in 0·01 M-potassium phosphate buffer, pH 7·0, was termed the *$(NH_4)_2SO_4$ precipitate fraction.*

This fraction was centrifuged for 10 min at 14,000 ***g***, the supernatant fluid decanted and centrifuged for 3 hr at 21,000 ***g***. The resulting pellet, containing the phage, was taken up in λ-dil. to a final volume that was 40% of that of the $(NH_4)_2SO_4$ precipitate fraction and after dialysis as above was termed the *21G fraction.*

Sufficient CsCl was added to the 21G fraction to make the phage suspension 41·5% CsCl (w/w), having a density at 4°C (ρ_4) of 1·46 g cm^{-3}. This solution was centrifuged 1 hr at 21,000 ***g*** and the clear liquid separating a small pellet and a floating gel removed and termed the *CsCl-1 fraction.*

The CsCl-1 fraction was centrifuged 42 hr at 27,000 rev/min in a Spinco model 30 rotor (maximum centrifugal force = 86,000 ***g***). Since λ and *λdg* have different buoyant densities in CsCl solutions (Weigle *et al.*, 1960), they form bands at different positions in the CsCl density gradient established in the centrifuge tube. This banding, as observed with the ultraviolet absorption optics of the Spinco model E centrifuge, is shown in Plate I. If a small hole is pierced in the bottom of the nitrocellulose centrifuge tube with an insect pin (size 00 or 0 pin attached to the tip of a soldering gun and heated prior to piercing), fractions can be collected, drop by drop, without appreciable disturbance of the CsCl and phage distributions. Fractions containing 80 to 90% of the active *λdg* phage were combined and dialyzed against 0·01 M-$MgSO_4$ in 0·01 M-potassium phosphate or 2-amino-2-hydroxymethylpropane-1:3-diol (tris-) buffers, pH 7·0, to yield *fraction CsCl-2.*

In some cases the *λdg* phage from fraction CsCl-2 were not dialyzed but were diluted in 41·5% CsCl solution and centrifuged 67 hr in a Spinco model SW25·1 swinging bucket rotor at 22,000 rev/min (maximum centrifugal force = 70,000 ***g***). Fractions were collected and dialyzed as described above to yield *fraction CsCl-3.*

A summary of the data obtained from the purification procedure is given in Table 1. Its effectiveness is indicated by the fact that the $(NH_4)_2SO_4$ precipitate fraction contains 60 times more 260 mμ-absorbing material and 400 times more protein per active λdg than does the purified CsCl-2 fraction. The 21,000 *g* centrifugation, addition of CsCl, and the preliminary 1 hr centrifugation, together, inactivate about one third of the phage. Since some, if not all, of the inactive phage fractionate with the active phage (e.g. in the CsCl gradient step), these purification factors are, therefore, minimal relative to total phage particles. Because of the uncertainty caused by this inactivation, a better criterion of the purity of the phage preparations in the latter stages of purification is given by the ratio of the absorbancy at 260 mμ to the protein concentration.

TABLE 1

The purification of λdg

Fraction	$\frac{\lambda dg}{\text{ml.}} \times 10^{-12}$	$\frac{\lambda dg}{\lambda}$	$\frac{\lambda dg/\text{ml.}}{As_{260}} \times 10^{-10}$	$\frac{As_{260}}{\text{mg prot./ml.}}$	% yield
Crude lysate	0·06	6·0	—	—	100
$(NH_4)_2SO_4$ precipitate	1·3	5·5	0·55	2·5	53
21G	2·4	5·5	2·8	4·0	42
CsCl-1	2·0	5·0	30	4·4	33
CsCl-2	14	12	42	13	26
CsCl-3	60	70	35	16	18

The method of preparation and assay of the fractions is given in "Materials and Methods." The reproducibility of the assay for λdg and λ is about 15% and that for the protein about 5%. As_{260} refers to the absorbancy at 260 mμ over a 1·00 cm light path after correcting for light scattered by the phage particles. The method of correction is given in the legend of Table 2. No values for the crude lysate are given in columns 4 and 5 because of the large contribution of the Difco bacto-tryptone to the protein and absorbancy values.

If one assumes that all inactive phage fractionate with active phage during centrifugation, then an approximate value of 5×10^7 for the gram-molecular weight of the DNA per λdg particle can be calculated from the data in Table 1, using a 260 mμ molar absorbancy index relative to phosphorus (or deoxyribose) of $6{\cdot}8 \times 10^3$ M^{-1} cm^{-1} (see Table 2). If, on the other hand, all of the λdg particles in the CsCl-2 fraction are assumed to be active, a value of 7×10^7 is found. These values are in agreement with the approximate gram-molecular weight of the DNA per λ particle of 5×10^7 which can be calculated from the data of Stent & Fuerst (1955) concerning the number of phosphorus atoms per λ that have a lethal effect when subject to ^{32}P decay if one assumes that the fraction of total phosphorus atoms exhibiting this effect is 0·1, the value found for phages T1, T2, T3, T5, and T7 (Stent & Fuerst, 1955). Since DNA constitutes 50% of the λdg mass (see "Experimental," Section (a)), the molecular weight of the λdg particle is twice the above values, i.e., about 1×10^8.

(h) *The preparation of λ and λdg DNA*

The DNA of λ or λdg phage was separated from the protein by a modification of the phenol method of Gierer & Schramm (1956). All operations were carried out at cold room temperatures of about 4°C. Fraction CsCl-2 was diluted in λ-dil. to a 260 mμ absorbancy of 10 (about 4×10^{12} λdg per ml.). Equal volumes of this phage suspension and freshly distilled phenol saturated with water at 4°C were mixed and agitated by hand for 1 min. The two phases were separated by centrifugation and the aqueous phase recovered. This process was repeated two more times, fresh water-saturated phenol being mixed with the recovered aqueous phase in each case. The three phenolic phase residues were extracted

serially with a volume of λ-dil. equal to one-fifth the volume of the phage suspension. The resulting two aqueous fractions were combined and dialyzed against 0·15 M-NaCl in 0·01 M-potassium phosphate buffer, pH 7·0, until all of the phenol was removed. The resulting clear, viscous solution is termed *λdg* DNA. It was stored, after freezing quickly in an ethanol-dry ice bath, at — 15°C and showed no loss of biological activity over the period of one year.

The DNA from helper phage λ, termed λ DNA, was also prepared according to the above procedure.

(i) *The nucleotide analysis of λdg DNA*

Fraction CsCl-3 was used as a source of *λdg* DNA for the determination of its nucleotide composition. Prior to isolating the DNA by the above phenol method, the phage suspension was dialyzed against 0·001 M-$MgSO_4$, 0·07 M-NaCl and 0·006 M-tris buffer, pH 7·5, and then treated with pancreatic DNase at a concentration of 1·0 μg per ml. for 1 hr at 37°C in order to hydrolyze any contaminating DNA. The phage were washed twice in the above tris medium by 3-hr centrifugations at 24,000 *g* and then treated with phenol. The phenol was removed by dialysis against 0·15 M-NaCl in 0·01 M-tris buffer, pH 7·5.

This DNA solution, containing 14 μmoles of phosphorus, was hydrolyzed to mononucleotides by the successive action of pancreatic DNase and venom phosphodiesterase (Koerner & Sinsheimer, 1957) according to the procedure of Lehman, Bessman, Simms & Kornberg (1958) with minor modifications in the concentrations of DNA and enzymes. The resulting digest contained 98 to 100% of the total phosphorus in the form of 5′-mononucleotides as indicated by the fact that this amount of phosphorus was released as orthophosphate after treatment of a portion of the digest with purified semen monoesterase (gift of Dr. L. Heppel) under conditions in which the contaminating phosphodiesterase activity of this enzyme preparation was not significant.

The 5′-mononucleotides were separated on a column of Dowex 1 (10×) acetate at 4°C using ammonium acetate buffers at pH 4·3 according to the method of Sinsheimer & Koerner (1951) except that 0·05, 0·25, 0·50, and 1·0 M buffer solutions were used to elute the 5′-monophosphates of deoxycytidine, deoxythymidine, deoxyadenosine, and deoxyguanine, respectively. The mononucleotides were identified by their absorption spectra and by their position in the elution diagram when compared to that of known nucleotides.

3. Experimental

(a) *The preparation and chemical analysis of λdg DNA*

Extraction of *λdg* with cold, water-saturated phenol releases the phage nucleic acid from its protein coat into aqueous solution. The chemical analysis of this material is given in this section. Its biological activity will be described in the following section.

The *λdg* phage that are used as the source of DNA in the phenol extraction ("Materials and Methods") are 50 ± 3% by weight DNA, as calculated from their phosphorus and deoxyribose content (Table 2). The remaining material in the phage is assumed to be protein, although protein analysis by the method of Lowry *et al.* (1951) yields the value of 62%. The inconsistency of this high protein percentage is most easily resolved by assuming that the bovine plasma albumin, used as a standard, is less reactive in this protein assay than is the average *λdg* phage protein.

The aqueous solution obtained after phenol extraction of the phage is termed *λdg* DNA and contains approximately 90% of the 260 mμ-absorbing material present in the phage. Since both the molar absorbancy index relative to phosphorus (α_p) at 260 mμ and the molar ratio of deoxyribose to phosphorus of the *λdg* DNA and of the phage are not significantly different (Table 2), the yield of DNA is also about 90%. On the other hand, less than 2% of the phage protein remains in the aqueous phase after phenol extraction. This is a maximum value determined by the significance level of the protein assay for the *λdg* DNA. The actual protein content of *λdg* DNA is

probably very much lower than this 2% value. Thus Dr. Simmons, in our laboratory, has recently found that in the phenol extraction of *λdg* labeled with ^{35}S only 0·1% of the phage sulfur appears in the aqueous phase.

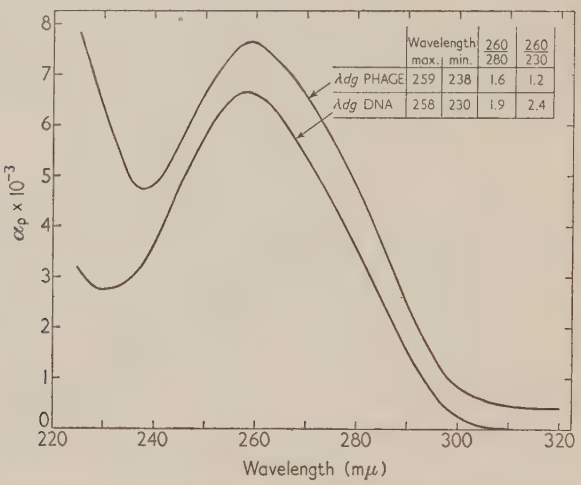

FIG. 2. The ultraviolet absorption spectra of *λdg* phage (fraction CsCl-3) and of *λdg* DNA at pH 7. The phage were suspended in 0·01 M-$MgSO_4$ and 0·01 M-potassium phosphate buffer and no correction was made for scattered light. The DNA was dissolved in 0·15 M-NaCl and 0·01 M-potassium phosphate buffer. α_p = molar absorbancy index relative to phosphorus.

TABLE 2

The chemical analysis of λdg phage and λdg DNA

Material	Percent phosphorus by weight	Deoxyribose to phosphorus molar ratio	α_p (260 mμ)	Molar ratio of deoxynucleotides
λdg phage	4·7	0·98	$6·8 \times 10^3$	—
λdg DNA	9·0	1·00	$6·6 \times 10^3$	A-1·00 T-1·00 G-0·95 C-0·94

The *λdg* phage (fraction CsCl-2) and *λdg* DNA were analyzed for phosphorus and deoxyribose after dialysis against water and freeze-drying, followed by drying over P_2O_5 *in vacuo* at room temperature. The *λdg* DNA was prepared by phenol extraction of phage fraction CsCl-2, except that for the analysis of deoxynucleotides fraction CsCl-3 was used (see "Materials and Methods"). The α_p at 260 mμ for *λdg* phage and *λdg* DNA was determined under conditions given in Fig. 2, except that a correction for the light scattered by the *λdg* phage has been made. The amount of scattered light was determined by the extrapolation to lower wavelength of a λ^{-3} curve fitted to the measured optical densities between 315 mμ and 400 mμ.

The ultraviolet absorption spectrum of *λdg* DNA (Fig. 2) is that expected of solutions of native DNA (Beaven, Holiday & Johnson, 1955) and exhibits a typical hyperchromic effect when treated with pancreatic DNase, the α_p at 260 mμ increasing 1·35-fold at pH 7 in 0·15 M-NaCl. Treatment of *λdg* DNA with pancreatic DNase also causes a decrease in the reduced viscosity from 65 dl. g^{-1} to less than 1 dl. g^{-1} when measured at 37°C in 0·14 M-NaCl, 0·015 M-sodium citrate at pH 7·6.

Hydrolysis of *λdg* DNA to mononucleotides can be accomplished by the successive catalytic action of pancreatic DNase and venom phosphodiesterase ("Materials and Methods"). Chromatography on Dowex 1 of the resulting digest ("Materials and Methods") showed that 98% of the 260 mμ-absorbing material and 97% of the phosphorus in the digest could be accounted for by the deoxynucleotides of adenine, thymine, guanine, and cytosine. No 5-hydroxymethyl-deoxycytidine-5′-monophosphate nor its *o*-mono- or diglucosylated derivatives was detected, although these nucleotides are separable by this technique (Lehman, personal communication) and would have been detectable if any one of them constituted greater than 0·3% of the mononucleotides in the digest.

TABLE 3

Components required for transformation

	Number of *Gal*$^+$
Complete system	2×10^4†
omit *λ*	0
omit *λdg* DNA	6
replace *λdg* DNA with *λ* DNA	10
replace *λdg* DNA with DNase-treated *λdg* DNA	6
omit *λdg* DNA and *λ*	0
omit bacteria	0

† A 10^{-2} dilution of the incubated mixture showed 204 *Gal*$^+$ colonies. The plates obtained from the undiluted mixture contained more than 5000 *Gal*$^+$ colonies and were therefore not countable.

The complete system contained 190 μg *λdg* DNA and $1{\cdot}5 \times 10^8$ λ-infected K12 $Gal^-{}_1Gal_4^-$ bacteria, 2 μmoles tris, pH 7·1, 2 μmoles $CaCl_2$, and 2 μmoles $MgSO_4$ in a total volume of 0·2 ml. The mixture was incubated 60 min at 37°C and then plated.

λ-infected bacteria were prepared by exposing $1{\cdot}5 \times 10^9$ bacteria to $1{\cdot}5 \times 10^{10}$ λ in P medium (made up to 0·01 M in $MgSO_4$) at 37°C for 15 min. At the end of the incubation the bacteria were centrifuged and resuspended in the diluent indicated above. Uninfected bacteria (for the line "omit λ") were similarly treated except that no phage was added.

DNase-treated *λdg* DNA was prepared by preincubating 380 μg *λdg* DNA with 0·2 μg pancreatic DNase at 22°C for 40 min in 0·2 ml. The inorganic components of this preincubation were the same as those in the complete system.

The amount of λ DNA or DNase-treated *λdg* DNA employed was also 190 μg.

The relative molar concentrations of the four mononucleotides present in the digest are given in Table 2. The expected equalities of adenine and thymine and of guanine and cytosine (Chargaff, 1955; Watson & Crick, 1953) occur. The fraction of bases which are guanine and cytosine in *λdg* DNA is 0·49. This quantity is a characteristic of the biological origin of a DNA preparation, varying from 0·3 to 0·7 for different microorganisms (Chargaff, 1955; Lee, Wahl & Barbu, 1956). The value for *λdg* DNA is equivalent, within experimental error, to that found for the DNA of a virulent mutant of λ (Lwoff, 1953) and of the λ host cell, *E. coli* K12 (Gandelman, Zamenhof & Chargaff, 1952).

The foregoing data show that the aqueous phase resulting from the phenol extraction of a *λdg* phage suspension is a solution of DNA. Less than 2% of the phage material in this solution is protein and, on the basis of its phosphorus and deoxynucleotide content, at least 95% is DNA.

(b) *Description of the transformation system and its requirements*

λdg DNA will transform galactose-negative (*Gal*$^-$) bacteria only if the bacteria have been exposed to ordinary λ, either simultaneously with or before the addition of the DNA. The requirement for added "helper phage," as it will be called in subsequent discussion, is shown in Table 3. Experiments which explore the role of helper will be presented in the next section; here, the point to be emphasized is that the level of transformation is at least 10^4-fold higher in the presence of helper than in its absence.

For these experiments the helper phage was propagated on Gal_4^- bacteria so that it could not mediate a transduction of the Gal_4^- recipient bacteria to *Gal*$^+$. The *Gal*$^+$ bacteria which arise in the absence of DNA, Table 3, are most likely due to reversions of the *Gal*$^-$ recipient bacteria which occur when it is growing on the assay plate.†

The transformation of *Gal* genes is a specific property of λdg DNA in the sense that neither λ DNA nor DNase-treated λdg DNA can replace λdg DNA. The results of both experiments are shown in Table 3.

The transforming activity of λdg DNA for *Gal*$^-$ mutants defective in the synthesis of galactokinase or in the synthesis of galactose phosphate uridyl transferase is similar. Thus 2·5 μg of λdg DNA produced 205 *Gal*$^+$ colonies from a Gal_4^-, transferase-less mutant and 130 *Gal*$^+$ colonies from a Gal_2^-, kinaseless mutant. The conditions of the experiment were those of the standard transformation assay described at the end of the next section. Therefore, λdg DNA, like λdg, carries both the gene for galactose phosphate uridyl transferase and the gene for galactokinase.

(c) *The quantitative determination of transforming activity*

The aim of the experiments described in this section was to develop an assay for the transforming activity of λdg DNA. These experiments also give some insight into the mechanism of the reaction between DNA and recipient cell and the role of helper.

(i) *Choice of helper phage*

Although wild type λ acts as helper, the variant λi^{434} acts more efficiently. At the same multiplicity of infection, 10 adsorbed phage per bacterium, 3·5 times more *Gal*$^+$ bacteria arose when λi^{434} was used as helper than when wild type λ was used. Phage λi^{434} was therefore adopted as helper for the standard transformation assay.

To avoid lysis of the recipient bacteria due to multiplication of helper phage and subsequent reinfection of the survivors during the assay, a *Gal*$^-$ lysogenic for λi^{434} was used as recipient.

(ii) *Time course of the reaction between λdg DNA and helper-infected bacteria*

If λdg DNA, helper phage, and recipient bacteria are mixed and DNase is added at various times later, the number of *Gal*$^+$ bacteria increases slowly to reach a plateau at 120 min, having attained one-half the maximum value at 40 min. The results of this experiment are represented in Fig. 3.

(iii) *Dependence on DNA concentration*

The number of *Gal*$^+$ bacteria obtained is proportional to the concentration of λdg DNA, as is shown in Fig. 4. This is true not only for an incubation of 120 min, a

† The data of Table 3 indicate that more revertants arise among the helper-infected bacteria than in the uninfected. This may be due to selection imposed by lysis and the products of lysis acting on the assay plates.

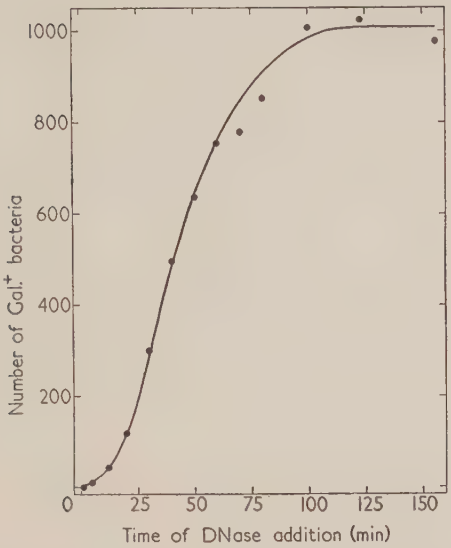

FIG. 3. Time course of the reaction between λdg DNA and helper-infected bacteria. DNA, helper, and bacteria were incubated together at 37°C in the following concentrations: λdg DNA—31 μg per ml., λi^{434} helper—$2{\cdot}6\times10^{9}$ per ml., and K12 Gal_4^- (λi^{434})—$4{\cdot}3\times10^{8}$ per ml. Media and diluents were the same as those described in the standard transformation assay. At the times indicated on the abscissa duplicate 0·3 ml. samples were removed from the incubation mixture and added to 0·1 ml. of 20 μg per ml. pancreatic DNase, incubated 5 min at 37°C and then plated. The number of Gal^+ bacteria given on the ordinate is the sum of that for two duplicate incubation mixtures.

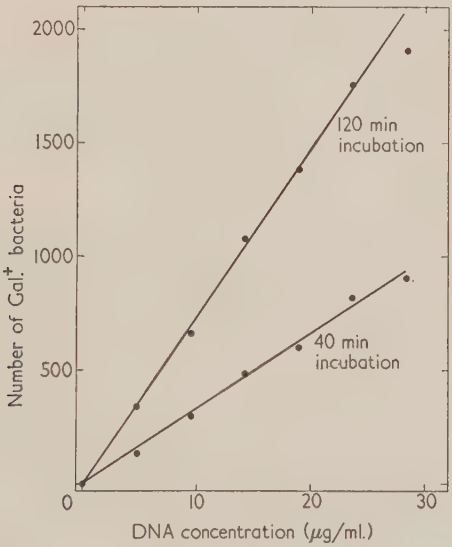

FIG. 4. Dependence on the concentration of DNA. Duplicate samples of λdg DNA at the concentrations indicated on the abscissa were incubated with K12 Gal_4^- (λi^{434}) at $5{\cdot}6\times10^{8}$ per ml. and λi^{434} helper at $2{\cdot}6\times10^{9}$ per ml. in a total volume of 0·3 ml. After 40 or 120 min at 37°C, 0·1 ml. pancreatic DNase at 30 μg per ml. was added, the mixtures incubated 5 min at 37°C and then plated. The number of Gal^+ recorded on the ordinate is the average of that for two duplicate incubation mixtures.

time which falls on the plateau of the time curve (Fig. 3), but also for an incubation of 40 min, the time at which uptake is one-half the maximum plateau value.

The specific transforming activity measured in this experiment after 120 min incubation is 75 *Gal*$^+$ per μg DNA. More recently specific activities of 8×10^5 *Gal*$^+$ per μg DNA have been obtained with the same DNA preparation but with different conditions of helper infection and DNA uptake.† The specific activity of whole λdg phage as measured in a transduction assay (see Table 1) is 2×10^9 *Gal*$^+$/μg DNA. Therefore, 4×10^{-4} of the activity present in λdg has been accounted for in terms of transformation by λdg DNA.

(iv) *Dependence on the concentration of helper-infected bacteria*

For a given amount of λdg DNA, the number of *Gal*$^+$ bacteria produced during a two-hour exposure is a linear function of the number of helper-infected bacteria,

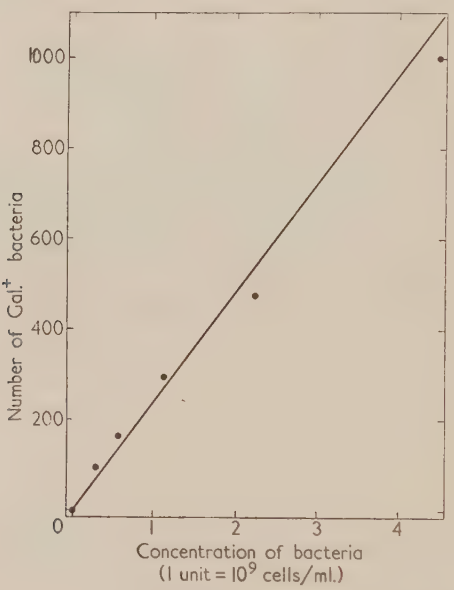

FIG. 5. Dependence on the concentration of helper-infected recipient bacteria. A number of 0·3 ml. incubation mixtures were made, each containing 8 μg per ml. λdg DNA and variable amounts of K12 *Gal*$_4^-$ (λi^{434}) ranging from $5{\cdot}7\times10^8$ to $4{\cdot}7\times10^9$ per ml. as indicated on the abscissa. The mixtures were completed by the addition of variable amounts of helper so as to keep a constant ratio of 9 phage per bacterium. After 120 min incubation at 37°C, 0·1 ml. pancreatic DNase at 20 μg per ml. was added, incubated 5 more min at 37°C, and then plated. The ordinate indicates the total number of *Gal*$^+$ bacteria on plates from two duplicate incubation mixtures.

as can be shown by (1) varying the concentration of bacteria, keeping the multiplicity of helper infection constant (Fig. 5), or (2) varying the multiplicity of helper infection for a given bacterial concentration.

In an experiment of the latter type, the number of *Gal*$^+$ bacteria is directly proportional to the multiplicity of infection for multiplicities below 0·1. Above 0·1 the

† The conditions were as follows. *Gal*$_1^-$ (λi^{434}) were infected with λi^{434} in P medium with $MgSO_4$ added to 0·01 M. The multiplicity of infection was 10. After 15 min incubation at 37°C the infected bacteria were centrifuged and resuspended in a solution containing 0·01 M-tris, pH 7·1, 0·01 M-$MgSO_4$, 0·01 M-$CaCl_2$. λdg DNA diluted in the same solution was added. Infected bacteria and DNA were incubated for 60 min at 30°C; then pancreatic DNase, 10 μg/ml. final concentration, was added and the mixture plated.

number of *Gal*$^+$ increases slowly with increasing multiplicity, reaching a maximum at a multiplicity of 4. The maximum is broad, extending from 4 to 14. The growth of the recipient bacteria is inhibited at multiplicities higher than 14.

(v) *Procedure for a standard transformation assay*

The following procedure was used to assay transforming activity in all subsequent experiments unless otherwise indicated. DNA, helper phage, and recipient bacteria were prepared as three separate reagents. The DNA was diluted to an appropriate concentration in 0·15 M-NaCl, 0·01 M-potassium phosphate buffer at pH 7·0. Helper λi^{434} was diluted to 10^{10} phage per ml. in λ-dil. The recipient bacteria Gal_4^- (λi^{434}) were grown in P medium containing 1·25 mg /ml. glucose to $1{\cdot}6\times10^9$ viable cells/ml., chilled, and then $MgSO_4$ was added to a final concentration of 0·01 M.

The assay was begun by mixing 0·1 ml. each of the diluted DNA, helper, and recipient bacteria. After 120 min at 37°C, 0·1 ml. of a 20 μg per ml. solution of pancreatic DNase was added, and the 37°C incubation was continued for 5 min more. Finally, 2 ml. of TB soft agar was added and the contents poured onto an EMB-galactose plate. The number of *Gal*$^+$ colonies was counted after 40 to 48 hr at 37°C.

Under the conditions of this assay, λdg DNA had a specific transforming activity of 75 *Gal*$^+$ per μg DNA. The mean deviation between duplicate assays with the same culture of bacteria was 15%, although the activities measured for the same DNA preparation using different cultures of recipient bacteria might differ by several-fold.

(d) *The nature of the active material*

The chemical and physical properties of λdg DNA are those of deoxyribonucleic acid. However, since only 4×10^{-4} of the activity present in whole λdg has been accounted for in terms of transformation by λdg DNA, it is possible that only 4×10^{-4} of the λdg complements of DNA, or even 8×10^{-5} since no more than 20% of the λdg phage particles register in a transduction assay, are still biologically active. If this were true, and if the loss in activity were due to a change in composition, the bulk chemical properties of λdg DNA might not represent those of the active material. It is necessary, therefore, to use other techniques to identify the active material.

(i) *The effect of anti-λ serum*

Since the active material is obtained from phage particles, it is pertinent to compare its properties with those of whole phage.

Neutralizing antibody reacts with the proteins of the phage tail to block some early step in the infection process, either adsorption or injection (Nagano & Oda, 1955). To see whether the active material in λdg DNA contains those structures of whole phage which react with neutralizing antibody, λdg DNA was treated with rabbit anti-λ serum.

The antiserum was first heated to 70°C for 45 min to denature serum nucleases. After heating, the serum contained less nuclease activity/ml. than would be equivalent to a solution containing 10^{-5} μg per ml. of crystalline pancreatic DNase, measured viscometrically with calf thymus DNA as substrate.

Results of this experiment are presented in Fig. 6. Whereas more than 95% of the transducing activity of λdg was neutralized in 160 min, no neutralization of the infectivity of λdg DNA was detected. The 10% decrease in infectious activity of λdg DNA which occurred in the presence of anti-λ serum occurred also in its absence.

To test the hypothesis that DNA might inhibit the neutralization reaction so that a small amount of whole *λdg* could escape detection in a large amount of *λdg* DNA, λ DNA was added to *λdg* and the course of neutralization of the mixture followed. The result, also presented in Fig. 6, was identical to the neutralization of *λdg* in the absence of added DNA.

The conclusion is that those structures of whole phage responsible for its reaction with neutralizing antibody are not necessary for the activity of *λdg* DNA.

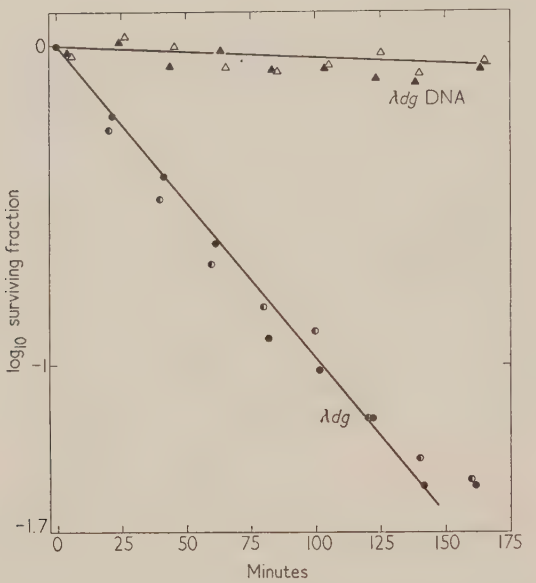

FIG. 6. The effect of anti-λ serum on the transducing activity of *λdg* and the transforming activity of *λdg* DNA.

1. (▲) 112 μg *λdg* DNA in 2 ml. anti-λ serum. The anti-λ serum had been diluted 1 to 33 in 0·15 M-NaCl, 0·01 M-potassium phosphate buffer, pH 7·0, heated 45 min at 70°C and further diluted 1500-fold in the same diluent.
2. (△) 112 μg *λgd* DNA in 2 ml. 0·15 M-NaCl, 0·01 M-potassium phosphate buffer, pH 7·0.
3. (●) $1{\cdot}6\times10^5$ *λdg* in 2 ml. anti-λ serum which had been treated as in 1.
4. (◐) $1{\cdot}6\times10^5$ *λdg* and 125 μg λ DNA in 2 ml. anti-λ serum which had been treated as in 1.

The four mixtures were incubated at 37°C and at the times indicated duplicate 0·1 ml. samples withdrawn and added to the standard transformation assay system as described in the text. A low concentration of antiserum was employed in the experimental mixtures so that neutralization of helper phage during the 2-hr assay would be negligible.

Initial values in the incubation mixtures were $7{\cdot}8\times10^3$ *Gal*$^+$ per ml. for 1 and 2; $1{\cdot}6\times10^4$ *Gal*$^+$ per ml. for 3 and 4.

(ii) *The effect of pancreatic DNase*

Whole phage is resistant to DNase because a shell of protein completely encases the nucleic acid. By contrast the transforming activity of *λdg* DNA is sensitive to this enzyme.

Sensitivity to pancreatic DNase was measured as a function of enzyme concentration from 10^1 to 10^{-4} μg per ml. For purposes of comparison whole *λdg* was treated with the same concentrations of enzyme. Results of this experiment, presented in Table 4, show that 10^{-4} μg per ml. of enzyme was sufficient to destroy 94% of the transforming activity in 30 min. It is unlikely, therefore, that inactivation is due to other enzymes contaminating the preparation of DNase.

The kinetics of inactivation of transforming activity and of decrease in viscosity were measured during exposure to 8×10^{-5} μg per ml. pancreatic DNase at 37°C. The results are plotted in Fig. 7. Transforming activity fell within 35 min to the limit of detectability which was for this experiment 0·4% of the initial activity. The viscosity, however, decreased more slowly; 70% remained after 50 min of enzyme treatment.

TABLE 4

Effect of DNase on the transducing activity of λdg and on the transforming activity of λdg DNA

DNase concentration (in mixture)	*Gal*+	
	λdg DNA	Whole *λdg*
μg/ml.		
10	0	245
1	0	229
10^{-1}	0	261
10^{-2}	0	197
10^{-3}	1	221
10^{-4}	16	220
0	264	246

Whole *λdg* or 4·7 μg *λdg* DNA and the indicated concentration of pancreatic DNase (in 0·01 M-$MgSO_4$, 0·01 M-potassium phosphate buffer, pH 7, 12 μg per ml. serum albumin) were incubated at 28°C in a total volume of 0·4 ml. After 30 min incubation 0·1 ml. of a λ suspension containing $4{\cdot}4 \times 10^{10}$ phage per ml. and 0·1 ml. of a TB culture of K12 Gal_4^- containing $7{\cdot}5 \times 10^9$ cells/ml. were added. The mixtures were incubated 20 min more at 28°C; then 0·2 ml. was withdrawn and plated.

The rate of inactivation of transforming activity is very close to that observed for *Hemophilus* transforming DNA (Zamenhof, Alexander & Leidy, 1953). Moreover, just as was observed for *Hemophilus* DNA, the decay of transforming activity is faster than the decay of viscosity.

(iii) *The effect of trypsin*

The active material in *λdg* DNA can be compared to the urea-treated T2 phage (Pi particles) which are capable of infecting protoplasts of *E. coli* but not the normal cells (Spizizen, 1957; Mahler & Frazer, 1959). The Pi particles are clearly differentiable from T2 phage but apparently still retain a structure consisting of other components in addition to the DNA.

The Pi particles exhibit a high and unique sensitivity to the action of trypsin. Whereas T2 phage are insensitive to 30 min exposure at 37°C to concentrations of trypsin as high as 100 μg per ml., Pi particles are over 90% inactivated at a trypsin concentration of 5×10^{-4} μg per ml. (Mahler & Frazer, 1959). When *λdg* DNA was treated with trypsin, no significant change in its activity could be demonstrated. Thus *λdg* DNA at a concentration of 130 μg per ml. was mixed at 37°C with from 1 to 90 μg per ml. of trypsin in either 0·05 M-potassium phosphate or tris buffer, pH 8·0. Samples of the reaction mixture taken at zero and at 60 min after the addition of the trypsin were diluted tenfold in 0·14 M-NaCl, 0·01 M-potassium phosphate buffer, pH 7·0, at 0°C and

then assayed for their activity. No significant difference (± 15%) in activity was observed between the zero and 60-min samples, the results being the same for control mixtures in which trypsin was absent.

While this result does not exclude the presence of some protein in the active *λdg* DNA molecules, it does indicate that trypsin-sensitive proteins, such as those present in Pi, are not necessary for activity.

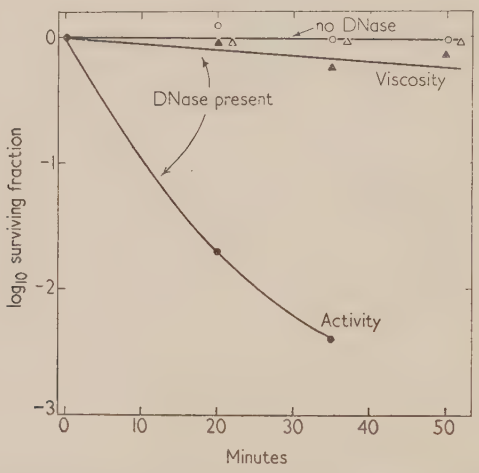

FIG. 7. Time course of the inactivation of *λdg* DNA by pancreatic DNase. Two incubation mixtures were prepared, each containing 210 μg *λdg* DNA, 20 μmoles $MgSO_4$, 10 μmoles potassium phosphate buffer, pH 7·0, and 20 μg serum albumin in a total volume of 1 ml. One mixture contained in addition 8×10^{-5} μg pancreatic DNase. After incubation for the times indicated at 37°C, 0·1 ml. samples were removed and diluted 1/20 in 0·15 M-NaCl, 0·01 M-potassium phosphate buffer, pH 7·2. Duplicate 0·1 ml. samples of the diluted mixture were then assayed by the standard transformation assay. Reconstruction controls carried out at the beginning and at the end of the experiment showed that the carry-over of 4×10^{-7} μg of DNase into the assay had no effect. As soon as the samples for the transformation assay were removed the remainder of the 20-fold dilution was used to measure the relative viscosity at 37°C.

(●) transforming activity, DNase present.
(○) transforming activity, DNase absent.
(▲) relative viscosity, DNase present.
(△) relative viscosity, DNase absent.

The initial values were 5×10^4 *Gal*$^+$ per ml. in the incubation mixture and a η_{sp} of 0·037 for the twentyfold dilution.

(iv) *The heat denaturation of λdg DNA*

A further criterion for defining the molecular nature of the infectious agent in the *λdg* DNA preparation is its stability to heat. As the temperature is raised, the DNA in an aqueous salt solution exhibits a transition from the relatively ordered structure of a double helix to a disordered coil (Rice & Doty, 1957; Doty, Boedtker, Fresco, Haselkorn & Litt, 1959). In the case of DNA from *Hemophilus influenzae* and *Diplococcus pneumoniae*, this transition results in the inactivation of that DNA with respect to genetic transformation (Zamenhof *et al.*, 1953; Marmur & Lane, 1960). The temperature at the midpoint of this transition, T_m, is a linear function of the guanine-cytosine content of a given DNA preparation (i.e. the fraction of bases which are guanine and cytosine) and lies between 84° and 96°C for the solutions of DNA from natural sources that have been examined (Marmur & Doty, 1959). With a guanine-cytosine content of 0·49, *λdg* DNA should have a T_m of about 89°C, the exact value depending upon the method used for measuring the transition.

Bacteriophage, on the other hand, are inactivated at considerably lower temperatures since the mechanism of their attachment and DNA injection into the host cell is dependent upon phage proteins which are more thermolabile than is the DNA. The characterization of the heat stability of the infectious activity of the *λdg* DNA preparation should therefore determine whether the DNA or some phage protein is the most thermolabile substance necessary for activity of the infectious agent.

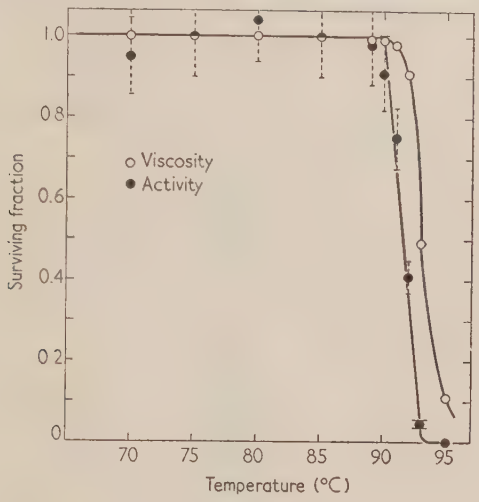

FIG. 8. The heat denaturation of *λdg* DNA with respect to transformation activity (●) and viscosity (○). The surviving fraction is the ratio of activity, or of viscosity, after heating for 60 min at the indicated temperature to that before heating. All activities and viscosities were determined at 37°C after cooling the heated material quickly in an ice-water bath. The dashed vertical lines associated with the activity values indicate the significance level ($\pm \sigma = \pm 10\%$) for this series of assays. Before heating, the 25 μg per ml. solution of DNA in 0·15 M-NaCl and 0·015 M-sodium citrate, pH 7·7, had an activity of 2×10^3 transformation units (*Gal*$^+$) per ml. and a reduced viscosity of 65 dl. g^{-1}.

The data presented in Fig. 8 indicate that the loss of transforming activity closely parallels the transition of the DNA structure as it is measured by the drop in viscosity attendant upon the loss of the double helical configuration. Thus, as the temperature is raised, the first significant loss in infectious activity and in viscosity occurs after one hour's exposure to a temperature between 90 and 91°C, the T_m values being 92°C for the activity and 93°C for the viscosity. In contrast, when complete *λdg* phage are heated under the conditions stated in Fig. 8, the surviving fraction of transducing activity is only 4×10^{-2} and 2×10^{-7} at 50° and 70°C, respectively.

It follows from these results that DNA is the most thermolabile substance in the active material of the *λdg* DNA preparation and further confirmation is given to the hypothesis that the proteins necessary for adsorption and DNA injection of *λdg* phage are not operative in this agent.

(v) *The buoyant density of the transforming agent in cesium chloride solutions*

Rolfe & Meselson (1959) and Sueoka, Marmur & Doty (1959) have shown that the buoyant density of DNA in cesium chloride solutions is related to its guanine-cytosine content (GC) by the following empirical equation:

$$\rho = 1{\cdot}66 + 0{\cdot}10\ (\mathrm{GC})\ \mathrm{g\ cm^{-3}}.$$

Since λdg DNA has a guanine-cytosine content of 0·49, it would be expected to exhibit a density of 1·71 g cm^{-3}. The λdg phage used in these experiments has a density of 1·49 g cm^{-3}, a value consistent with its 50% DNA content if one assigns a density of 1·3 g cm^{-3} to the phage protein. Thus one would expect that any product of λdg phage degradation would exhibit a density determined by its protein to DNA mass ratio. Provided the active material in the λdg DNA preparation is large enough to form a band in a cesium chloride gradient, the density at the band position should yield a measure of the protein to DNA mass ratio of the active unit.

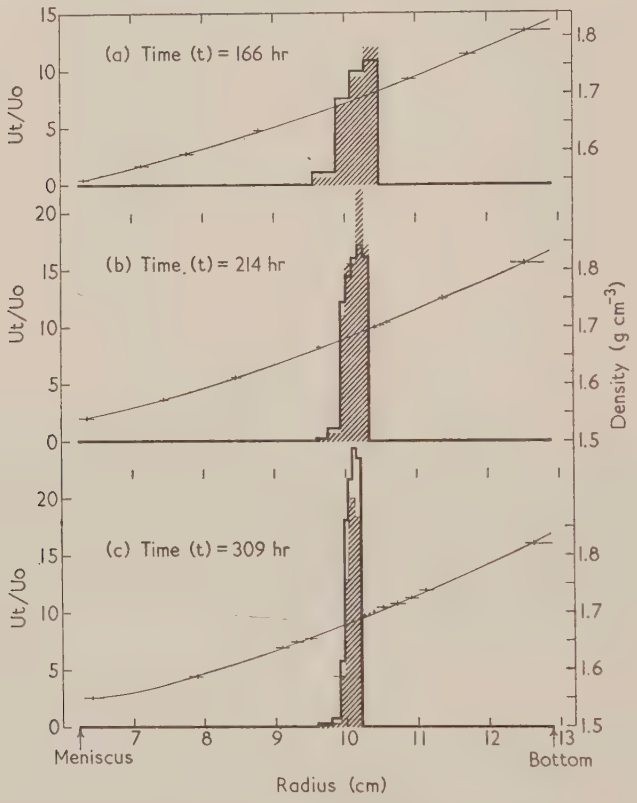

Fig. 9. Centrifugation of λdg DNA in a cesium chloride gradient.

(1) Left-hand ordinate. The ratio of 260 mμ absorbancy (heavy solid line) or of transformation activity (shaded) at time (t) to that at the beginning of centrifugation ($t = 0$).

(2) Right-hand ordinate. The density at 4°C of fractions whose size is indicated by the short horizontal lines, the short vertical line being at the mean radius of each fraction. Density determinations are accurate to $\pm$ 0·0015 g cm^{-3}.

(3) Conditions: At $t = 0$ hours, CsCl = 5·24 M; tris = 0·023 M; pH = 8·6; $\rho_4 = 1{\cdot}661$; 260 mμ absorbancy = 0·77; activity = 8×10^3 transformation units per ml.; and temperature = 4°C. The solution was centrifuged in a Spinco SW 25·1 swinging bucket rotor at $22{\cdot}5 \times 10^3$ rev/min and the temperature at the end of each run was 5 $\pm$ 1°C.

(4) Percent recovery: 260 mμ absorbancy = (a) 101, (b) 103, and (c) 98; transformation activity = (a) 96, (b) 108, and (c) 86.

To this end, λdg DNA in cesium chloride solutions was centrifuged for various times under the conditions indicated in Fig 9. Fractions were collected from the centrifuge tubes by the technique employed in the cesium chloride gradient purification of λdg phage ("Materials and Methods"). Analysis of these fractions for their ultraviolet

absorption spectrum, their density at 4°C, and their transforming activity after dialysis against 0·15 M-NaCl containing 0·01 M-potassium phosphate buffer, pH 7·0, yielded the results given in Fig. 9 and Table 5.

Since the recovery of absorbancy at 260 mμ and of transforming activity after each centrifugation was complete within the experimental error of the techniques employed (± 15% for activity and ± 4% for the absorbancy), and since better than 98% of the recovered value was found in the visually obvious band regions of Fig. 9, it is sufficient to limit the analysis of the fractions to this band region.

TABLE 5

Comparison of the DNA and transforming activity distributions during centrifugation in a cesium chloride gradient

Time (hours)	Mean density (g cm^{-3})		Standard deviation (g cm^{-3})		Density gradient (g cm^{-4})
	DNA	activity	DNA	activity	
166	1·691	1·692	0·0084	0·0080	0·045
214	1·687	1·687	0·0061	0·0056	0·048
309	1·685	1·685	0·0042	0·0043	0·048

The distributions analyzed are those shown in Fig. 9. The DNA was measured by its absorbancy at 260 mμ.

The mean density and standard deviation of density for the activity and absorbancy distributions (Table 5) are not significantly different from each other at each centrifugation time. Furthermore, there are no significant differences in the transforming activity per unit absorbancy at 260 mμ (specific activity) among the various fractions and that of the input material, except for one to two per cent of total recovered activity occupying the lowest density region of the bands, in which case the specific activity was about 60% of that found in the other fractions. The discrepancy between the observed density of 1·685 g cm^{-3} and the value of 1·71 g cm^{-3} predicted above is due to the different conditions employed in this experiment from those used in determining the empirical equation relating density to guanine-cytosine content, since the DNA used in this experiment yielded a buoyant density of 1·708 ± 0·001 when measured under the latter conditions (Rolfe, personal communication).†

These results indicate that the active molecules have the same protein to DNA mass ratio as that of the bulk of the DNA. As indicated in Section 1 the value of this ratio is less than or equal to 0·02 on the basis of the protein assay of Lowry *et al.* (1951) and less than or equal to 0·001 on the basis of sulfur content. Furthermore, from the equality of the activity and DNA distributions at the various times of centrifugation, it is concluded that the size of the active molecules is of the same order of magnitude as that of the DNA molecules. Thus the conclusion derived from this experiment and

† The significant difference in the conditions is that the CsCl used in our experiments was impure, containing 4·11% rubidium chloride (see "Materials and Methods"), whereas that used by Rolfe & Meselson (1959) was 99·9% CsCl (Maywood Chemical Works). Thus we have recently obtained a mean buoyant density of 1·71 for *λdg* DNA under the conditions stated in Fig. 9 when using the purer CsCl.

consistent with the previous results is that the active material consists of molecules of DNA in aqueous solution.

(e) *Cotransformation of Gal and phage genes*

The galactose-positive bacteria produced by exposure to *λdg* DNA have two characteristic properties. First, they are unstable. Isolated colonies, previously purified by at least two single colony isolations, contain one galactose-negative bacterium per several hundred galactose-positive bacteria. Secondly, they produce lysates after irradiation with u.v. light which are capable of transducing the gene for galactose-l-phosphate uridyl transferase at high frequency.

In both respects these strains behave like *Gal*$^-$ (*λdg*). When a *λdg* prophage is lost, which happens about one in 10^3 cell generations, the host becomes *Gal*$^-$ (Morse *et al.*, 1956; Campbell, 1957). U.v. irradiation of bacteria doubly lysogenic for *λdg* and *λ* induces the production of both *λ* and *λdg* in roughly equal amounts. The lysate produced will, therefore, transduce with high frequency.

A quantitative comparison of segregation and production of *λdg* for a series of galactose-positive strains produced by exposure to *λdg* DNA and those produced by transduction with *λdg* is presented in Table 6. The similarity of the results obtained

TABLE 6

Segregation of Gal$^-$ *and phage production by Gal*$^+$ *transformants*

Donor	Segregation of *Gal*$^-$ bacteria		Production of *λdg*			
				number having *λdg*/*λ* values in the range		
	number tested	number segregating	number tested	0·5–2·0	2·1–4·0	4·1–6·0
λdg DNA	56	52	21	10	9	2
λdg	56	51	18	9	6	3

Using the procedure of the standard DNA transformation assay, galactose-positive bacteria were isolated after exposure to *λdg* DNA or to *λdg*. From each of the two series a number of galactose-positive bacteria were picked and streaked out on EMB-galactose plates. To avoid bias in the selection of colonies, all galactose-positive bacteria within an arbitrary sector of each of two plates were picked. A single galactose-positive colony was picked from each streak and restreaked so as to obtain more than 500 isolated colonies. These streaks were examined for galactose-negative colonies. From streaks which showed at least one galactose-negative colony a single galactose-positive colony was picked and grown in H-glucose liquid medium and a lysate prepared by u.v. induction. The lysates were assayed for their plaque-forming and transducing activities using the assays for *λ* and *λdg* described in the section on "Materials and Methods." It should be noted that the helper phage for both series of experiments was *λi*^{434}.

with *λdg* DNA and *λdg* supports the hypothesis that bacteria transformed by *λdg* DNA become galactose-positive because they are lysogenic for *λdg*. In both experiments *λi*^{434} was helper. All of the lysates examined contained both *i*$^{\lambda}$ and *i*^{434} phages; therefore, the *dg* prophage could be either *i*$^{\lambda}$ or *i*^{434}. It is conceivable that only the segment bearing the galactose gene(s) came from *λdg* DNA and the rest of the *λdg*

genome necessary for the formation of a complete prophage was supplied by recombination with the helper phage. This possibility was examined by using *λdg* derived from wild type λ as the source of *λdg* DNA and helper phage marked in the *c* region and at the *mi* locus. The *c* region and *mi* are on the same side of the defective region; *c* is 5 map units and *mi* 10 map units from the nearest end of the defective region (see Fig. 1) (Arber, 1958). Genes present in *λdg* can be detected among the active λ produced by bacteria lysogenic for both *λdg* and λ because genetic recombination occurs between λ and *λdg* during their multiplication together in the same cell.

In the first experiment *λdg* DNA was employed with λ co_1 *mi* as helper and nonlysogenic K12 Gal_4^- as recipient. The co_1 marker is in the *c* region. Control transductions were performed in which *λdg* replaced the DNA extract, the experiments being otherwise identical. Galactose-positive bacteria were picked and purified by three single colony isolations in EMB-gal plates, grown in H-glucose medium, induced with u.v. light, and plated on sensitive bacteria. These plates were examined for the presence of co_1, co_1^+, *mi*, and mi^+. Approximately 200 plaques were examined from each lysate. The results are presented in Table 7.

TABLE 7

Cotransformation of Gal⁺ and phage genes by λdg DNA

Agent	Number of *Gal*⁺ containing:					Total
	co⁺ and *mi*⁺	*co*⁺ only	*mi*⁺ only	neither	i^λ	*Gal*⁺ tested
λdg (1)	17	0	1	3		21
λdg DNA (1)	24	2	2	27		55
λdg DNA input to CsCl, Fig. 9 (2)					1746 (97%)	1808
Peak fraction, 214 hr, Fig. 9b (2)					1935 (> 99%)	1942

(1) $2{\cdot}5 \times 10^8$ *λdg* or 12 μg *λdg* DNA were added to 2×10^8 K12 Gal_4^- and 4×10^9 λ *co mi* and the mixtures plated. Isolated *Gal*⁺ colonies were picked and restreaked twice. The purified strains were induced to produce phage by exposing them to u.v. light, and the active phage produced examined by plating on C600. λ *co mi* forms small clear plaques while *co*⁺ causes turbid plaques and mi^+ large plaques. At least 500 plaques were examined from each lysate. When either *co*⁺ or mi^+ was present, it was present in roughly 25% of the active phage.

(2) Galactose-positive bacteria which had arisen from a standard transformation assay and were growing on the EMB-galactose plate were scored for the presence of a halo of lysis. A halo would indicate that the bacteria were producing phage with the immunity specificity of λ, symbolized i^λ. See the text for a more complete explanation of the technique.

In the control series with *λdg*, 17 out of 21 or 81% of the galactose-positive bacteria isolated had both alleles of *λdg*, co_1^+, and mi^+. Among the *Gal*⁺ arising from DNA-treated cells, 24 out of 55 or 44% had both co_1^+ and mi^+.

In another experiment the *c* gene was detected by making use of the fact that it controls the specificity of immunity. Transformation with *λdg* DNA was carried out with λi^{434} as helper and K12 Gal_4^- (λi^{434}) as recipient, plating finally on EMB-galactose agar. Normally the untransformed K12 Gal_4^- (λi^{434}) recipient bacteria grow to form a confluent, white background on the EMB plates. Because bacteria carrying a prophage with the immunity gene of λ, symbolized i^λ, will produce some active phage with the immunity specificity of λ, a halo of lysis of the background will

surround a galactose-positive colony if it has received the i^{λ} gene in addition to a galactose gene from the *λdg* DNA. The EMB plates are therefore scored for the total number of galactose-positive colonies and for the number of galactose-positive colonies with a halo of lysis.

The results of this experiment are presented in Table 7 also. The i^{λ} gene was present in at least 97% of the galactose-positive bacteria.

It should be pointed out that both experiments give minimum estimates of the frequency of cotransfer of *Gal* and phage genes because loss of prophage genes is known to occur during growth of polylysogenic bacteria (Appleyard, 1954; Campbell, 1957). The lower frequency of cotransfer in the first experiment might be explained by the greater number of bacterial divisions occurring between exposure to DNA and testing.

Thus bacteria which become galactose-positive because they have been transformed by *λdg* DNA have a high probability of receiving both the *c* and *mi* genes as well. The interval between *mi* and the farthest end of the defective region is about 90% of the known map of *λ* (see Fig. 1). If it is assumed that cotransformation means that *Gal*, *c*, and *mi* are on the same DNA molecule, this molecule must include essentially all of the *λdg* genome.

4. Discussion

A comparison of some of the chemical and biological properties of the *λdg* DNA system with those of the *D. pneumoniae* and *H. influenzae* transformations, the *λdg* transduction, and with urea-treated T2 phage (Pi particles) is given in Table 8. The active material in *λdg* DNA is similar to the *Pneumococcus*- and *Hemophilus*-transforming DNA in that their physical and chemical properties define them as molecules of DNA in aqueous solution. This is not the case for the Pi particles which evidently retain appreciable T2 protein as necessary components for infection.

The *λdg* DNA system is appreciably less efficient than the other systems listed in Table 8 in terms of the number of transformed cells per μg of DNA added. This difference is more marked relative to classical transformation if the measure of efficiency is the number of transformed cells per phage- or bacterial-equivalent of added DNA, this being the closest approximation that can presently be made to the number of cells transformed per donor genome. Because of the twenty-five- to sixty-fold greater amount of DNA per bacterial cell than per *λdg*, the *λdg* DNA system is, on this basis, 10^{-3} to 10^{-4} as efficient as classical transformation. The lower efficiency might be due either to the nature of the recipient cells, the DNA, or both.

The most important factor in determining the competence of recipient cells in the *λdg* DNA system is their infection with helper phage. If it is assumed that the active DNA molecule contains essentially all of the *λdg* genome, one of the roles played by helper phage in transformation should be the same as that which it performs in transduction: it should alleviate the *dg* defect in the reactions leading to lysogenization. However, the effect of helper phage in the transformation system is to increase the efficiency by a factor of at least 10^4 while having only a twenty-fold effect in the *λdg* transduction. Consequently it must be supposed that helper phage also supply a function present in the whole *λdg* phage, but absent in the *λdg* DNA. This function could involve the adsorption of the DNA to the cell surface, the penetration of that surface, or the several reactions by which the injected phage chromosome becomes prophage. One would expect that *λdg* DNA labeled with ^{32}P could be used to further explore these possibilities.

TABLE 8

A comparison of the properties of the D. pneumoniae *and* H. influenzae *transforming DNA, λdg DNA, λdg phage and Pi particles*

Property	Transforming DNA(1)	*λdg* DNA	*λdg* phage	Pi particles(2)
I. *Chemical*				
(1) effect of DNase	$< 10^{-4}$ μg/ml. inactivates	$< 10^{-4}$ μg/ml. inactivates	none	$> 10^{-2}$ μg/ml. partially inact.
(2) effect of trypsin	none	none	—	$< 10^{-4}$ μg/ml. inactivates
(3) effect of antiserum	—	none for anti-λ	anti-λ inactivates	anti-T2 inactivates(3)
(4) thermolability	same as DNA	same as DNA	more labile than DNA	more labile than DNA
(5) density, CsCl	—	same as DNA (1·71)	less than DNA (1·5)	CsCl inactivates
(6) protein content of active preparation	< 0·02%	< *ca.* 1%	50%	—
II. *Biological*				
(1) efficiency: no. of transformed cells per μg DNA added(4)	1×10^8	1×10^6	2×10^9	2×10^7
(2) DNA per donor bacterium or phage	$2–5 \times 10^{-9}$ μg per bact.(5)	8×10^{-11} μg per phage(6)	8×10^{-11} μg per phage(6)	2×10^{-10} μg per phage(7)
(3) Percent of genome transferred	*ca.* 1(8)	*ca.* 100	100	100

(1) Zamenhof (1957).
(2) Mahler & Frazer (1959).
(3) Whether anti-T2 or DNase in the serum caused the inactivation was not determined (Mahler & Frazer, 1959).
(4) This efficiency of transforming principle and of *λdg* DNA varies with recipient cell concentration. The figure for transforming principle is for *H. influenzae* at $1·6 \times 10^9$ cells per ml. (Goodgal & Herriott, 1957) and that for *λdg* DNA is with 1×10^9 helper-infected cells per ml. under the most recent assay conditions (see "Experimental," Section 3c). The value for *λdg* transduction is taken from Table 1, fraction CsCl-2. Pi particles were assayed at 2×10^9 protoplasts per ml., and the efficiency was calculated assuming 2×10^{-16} g DNA per T2 particle and no loss of DNA during preparation of Pi particles (Mahler & Frazer, 1959). In this latter case, one plaque is taken as one "transformed" cell.
(5) *H. influenzae*: 2×10^{-15} g/cell (Zamenhof *et al.*, 1953).
D. pneumoniae: 2×10^{-15} g/cell (Fox, 1957).
D. pneumoniae: 5×10^{-15} g/cell (Lerman & Tolmach, 1957).
(6) See " Materials and Methods," Section 7.
(7) Herriott & Barlow (1952); Stent & Fuerst (1955).
(8) The value must be considerably less than 100% since most known transforming factors are transferred independently (Hotchkiss, 1954). An estimate of this value can be made from the molecular weight of $1·5 \times 10^7$ calculated for the streptomycin-resistance factor of *H. influenzae* (Goodgal & Herriott, 1957) and the value of 2×10^{-15} g DNA, or 80 DNA molecules of molecular weight $1·5 \times 10^7$ per cell. Assuming one genome contains these 80 molecules, genetic transfer by one molecule would amount to the transfer of about 1% of the genome.

The unique features of the *λdg* system which most easily distinguish it from transformation in *Pneumococcus* and *Hemophilus* are the small size of the donor genome and the knowledge of its structure from phage genetics. These characteristics are reflected in the fact that the *Gal*$^+$ transformants always have *c* and in half of the cases *mi* which come from the DNA. Since the linkage between *λdg* and *c* has been observed at DNA concentrations as low as 0·1 *λdg*-equivalents per recipient bacterium, and since the number of *Gal*$^+$ is a linear function of the DNA concentration, the simplest interpretation is that the active molecules carry *dg*, *c*, and *mi*. Assuming that the entire *dg* region, as mapped by Arber (1958), is present, and that the segments between *dg* and *c*, and between *c* and *mi* are also present, this represents 90% of the *λdg* map (Fig. 1). If the *λdg* genome contains all of the DNA in the phage, the active molecules should have a molecular weight of 5×10^7 ("Materials and Methods," Section (g)).

In *Pneumococcus* and *Hemophilus*, on the other hand, most of the known transforming factors are transferred independently. This is to be expected in view of the fact that a donor bacterium contains the equivalent of 25 to 60 DNA molecules of molecular weight 5×10^7 whereas the molecule of transforming DNA is certainly not more than 5×10^7 (see footnote 8, Table 8). Therefore, the *Pneumococcus*- and *Hemophilus*-transforming DNA's are heterogeneous in that different molecules represent different segments of the bacterial chromosome. *λdg* DNA does not exhibit this form of heterogeneity since the active molecules comprise the entire donor phage chromosome. The absence of this form of heterogeneity and the availability of a variety of markers, all linked, should make *λdg* DNA useful for an analysis of the chemical structure of the phage chromosome.

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Studies on Basic Proteins from Ribosomes of Buds of Pea Seedlings †

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Ribosomes from buds of pea seedlings were extracted with cold dilute hydrochloric acid. Starch gel electrophoresis of the extract revealed a large number of proteins with isoelectric points around pH 8. At pH 4·2, at least sixteen zones of different intensities were resolved, forming a complex electrophoretic pattern. This pattern was obtained from several preparations of pea bud ribosomes of differing purity and was essentially unchanged after pervaporation, dialysis, prolonged storage, or treatment with 4 M-urea. Acid extracts of the nuclear fraction were electrophoretically distinct from the acid-soluble ribosomal proteins. The N-terminal amino acids of the extracted proteins were similar to those of the intact ribosomes indicating that no significant hydrolysis occurred during extraction. Nitrogen, phosphorus, and ultraviolet spectral analyses showed that the acid-soluble proteins were free of RNA and represented about 29% of the protein of the ribosomes. On ultracentrifugation the acid-soluble protein fraction appeared polydisperse, with a mean sedimentation coefficient of about 0·9 S, and an average molecular weight of 18,000.

1. Introduction

Intact ribosomes from a variety of biological sources have been subjected to extensive physical and chemical study. In general, these studies indicate a basic similarity of the particles from all sources and homogeneity from any one source, e.g. pea seedlings (Ts'o, Bonner & Vinograd, 1956). However, since ribosomes have been implicated in the final stages of protein synthesis (e.g. Raacke, 1959), it is reasonable to expect that despite physical similarity, these structures, even within the same cell, are chemically heterogeneous. Such heterogeneity should be revealed in studies on components of the particles but despite some investigations (Ts'o, Bonner & Vinograd, 1958; Elson, 1958) the nature of the proteins and RNA within ribosomes remains relatively obscure.

During the course of cytochemical investigations using the fast-green histone stain of Alfert & Geschwind (1953) we consistently obtained a mild cytoplasmic staining reaction in meristematic plant cells after removal of nucleic acids. Since such cells are rich in ribosomes (Lund, Vatter & Hanson, 1958; Setterfield, Stern & Johnston, 1959) this cytoplasmic staining may be due to basic proteins present in the ribosomes and bound to the RNA in a manner analogous to the binding of histone to DNA. This paper reports experiments in which the hydrochloric acid extraction method for histones was applied to ribosomes of pea seedling buds and the extracted protein fraction analysed by starch gel electrophoresis and other methods. The results show that the ribosomes contain a complex mixture of acid-soluble proteins which may provide a basis for heterogeneity of the particles.

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2. Materials and Methods

Ribosomes were prepared from the apical buds of pea seedlings. Seeds (variety Arthur) were soaked for 2 hr and germinated in damp vermiculite in darkness for 6 to 8 days. The apical buds were then removed with a minimum of stem, ground in a mortar and fractionated by differential centrifugation. Usually about 80 to 120 g fresh weight of buds were used in each experiment. The fractionation procedure was that of T'so *et al.* (1956) using a no. 40 rotor on a Spinco model L ultracentrifuge. In most cases the fractionation was taken to the level of "DC-2 particles" (Ts'o *et al.*, 1956) with two centrifugations at 40,000 rev/min, while in one experiment additional centrifugations at 15,000 rev/min and 40,000 rev/min yielded "DC-3 particles" (in nomenclature consistent with that of the above authors). In one experiment half the ribosomal pellet was resuspended in 0·3% deoxycholate (DOC) instead of water after the first centrifugation at 40,000 rev/min (DC-1 particles). Following the second 40,000 rev/min centrifugation these particles were again resuspended in water. In each experiment the volume of the preparation of particles was reduced during centrifugation and resuspension to 10 to 12 ml.

As a source of histone in control experiments the "nuclear" fraction of Ts'o & Sato (1959) was prepared. According to these authors this fraction contains 98% of the DNA from pea seedlings.

Protein was extracted from the ribosomes (or from the nuclear fraction in the case of histone) by the procedure used by Neelin & Butler (1959) for histone extraction. All operations were at 0° to 5°C. Molar HCl was added dropwise to the suspension to give a pH of 1·7 and the preparation was blended for 30 sec at top speed on a Virtis homogenizer and centrifuged for 1 hr at 40,000 rev/min. Dilute NaOH was added to the resulting supernatant to give pH 3·5 to 4·0. This solution was either used directly for electrophoresis or concentrated two- to fourfold by pervaporation at 4°C. When electrophoresis was performed at pH 6 or above the solution was dialysed overnight at 0°C against the buffer used for electrophoresis.

Horizontal starch gel electrophoresis (Smithies, 1955) was performed using conditions similar to those described for analysis of calf thymus histone (Neelin & Neelin, 1960). Electrophoresis was continued for 8 hr in a voltage gradient of 4·0 ± 0·7 v/cm of gel length and a current of 3·5 mA/cm of gel width. The gels (26 × 2·0 × 0·6 cm) consisted of 15·4 g of "Starch–Hydrolysed for gel electrophoresis" (Connaught Medical Laboratories, Toronto) in 100 ml. of buffer of ionic strength 0·020. Formate, acetate, cacodylate, phosphate, veronal, borate, tris, and glycine buffers were used, and pH values were determined on the starch buffer mixtures. In a few experiments gels were made up in buffered 4 M-urea, in which case a voltage gradient of 3·5 ± 0·5 v/cm was used. For introduction into the gels, samples were suspended in a thick starch paste (arrowroot or potato starch powder) in slots usually 4 mm wide. In a few cases, with unavoidably dilute samples, the slot width was increased to 8 mm even though this gave noticeably inferior resolution. After electrophoresis duplicate halves of the gels were stained with amido black B and nigrosin respectively.

N-terminal residues of the intact ribosomes and of the extracted ribosomal proteins were determined by the 1-fluoro-2,4-dinitrobenzene (FDNB) method of Sanger (1945) with conditions of reaction, hydrolysis, and chromatographic separation as described previously (Neelin & Neelin, 1960). Examination for water-soluble amino acids was carried out as suggested by Levy (1955). To avoid gelation in guanidine hydrochloride, ribosomes were reacted with FDNB in two volumes of methanol.

Total nitrogen of the ribosomes and ribosomal proteins was determined by the micro-Kjeldahl method (Ma & Zuazaga, 1942) and total phosphorus by the method of King (1932). Ultraviolet spectra were obtained with a Cary quartz spectrophotometer, model 11 M. The anthrone carbohydrate test was applied by adding a few drops of the sample to a solution of 0·5% anthrone in conc. H_2SO_4 and observing the green colour at the interface.

Ultracentrifuge analyses of the ribosomes and ribosomal proteins were performed in a Spinco model E instrument. The average molecular weight of the ribosomal proteins was estimated by the Archibald method using the procedure given by Champagne & Smith (1960) as modified later by Smith (in preparation). The protein solution was dialysed against 0·1 M-acetate buffer pH 4·3, for 24 hr and centrifuged in a synthetic boundary cell

at 20,410 rev/min followed by 42,040 rev/min. To estimate initial concentration of protein the cell with sample was shaken, a layer of buffer added and centrifugation continued at 20,410 rev/min. Measurements for calculations were obtained from tracings of projected enlargements of the schlieren patterns.

For electron microscopy pellets of sedimented ribosomes were fixed in 2% osmium tetroxide buffered at pH 7·2 (Palade, 1952), embedded in methacrylate and sectioned. Sections were cut so as to pass through the entire thickness of the pellet and were stained with lead (Watson, 1958).

3. Results

(a) *Purity of ribosomes*

Typical results of electron microscope and ultracentrifuge analyses of DC-2 preparations of ribosomes are shown in Plate I. The results are in agreement with those of Ts'o *et al.* (1956). In the electron microscope the entire pellet appeared to be composed of particles 100 to 200Å in diameter, which stained readily with lead hydroxide (Plate I(a) and (b)). No significant amount of contaminating non-particulate material was encountered. The ultracentrifuge patterns show that about 95% of the sedimentable material of the fraction consisted of particles with an uncorrected sedimentation coefficient of 76 s (Plate I(c)) while the remaining material was in the 24, 40, and 120 s peaks reported by Ts'o *et al.* (1956). In some experiments ribosomes were stored for several days at 0°C and Plate I(c) indicates that negligible deterioration of the particles occurred during this time.

(b) *Starch gel electrophoresis*

The results of starch gel electrophoresis of hydrochloric acid extracts of ribosomes are presented in Plates II and III. The pH values ranged from 3·3 to 8·9 and the patterns represent materials migrating toward the cathode. No migration of material to the anode was detected.

At pH 5·2 and lower, eight main zones and a number of minor components could be observed (Plate II(a) to (d)). The optimum pH, combining good mobility and resolution, was pH 4·2, at which a minimum of sixteen zones were resolved. These zones are numbered in order of decreasing mobility (Plate II(b) and (c)). At the low pH values zone 1 migrated to the cathode wick and lies beyond the limits of the figures. Zones 12, 15, and 16 stained strongly with nigrosin but were only barely visible with amido black. Below pH 4·2 the patterns showed little difference except for slightly increased migration (Plate II(a)). Migration decreased markedly above pH 6 (Plate II(e) to (k)) and a sharp zone, stained strongly by nigrosin but not by amido black, became evident. This zone retained approximately the same mobility from pH 6 to pH 8·9 (Plate II(f), (h) and (j)). The remaining zones migrated only slightly above pH 6·8, and failed to enter the gel at about pH 8·5 (Plate II(h), (j) and (k)). These results indicate that the average isoelectric point of the ribosomal proteins was about pH 8.

Electrophoresis at pH 4·2 (acetate buffer) was chosen as a standard assay condition for further experimentation. The specific electrophoretic pattern at this pH (Plate II(b) and (c); Plate III(a)) was obtained, with only minor variations, from hydrochloric acid extracts of 5 separate preparations of ribosomes derived from buds of seedlings from 6 to 8 days old. The variations in the patterns were similar to those obtained in replicate runs of the same preparation and were undoubtedly due to small uncontrolled variables in the electrophoretic procedure. The pattern from DC-3 particles was essentially the same as that from DC-2 particles. Treatment of the ribosomes with 0·3%

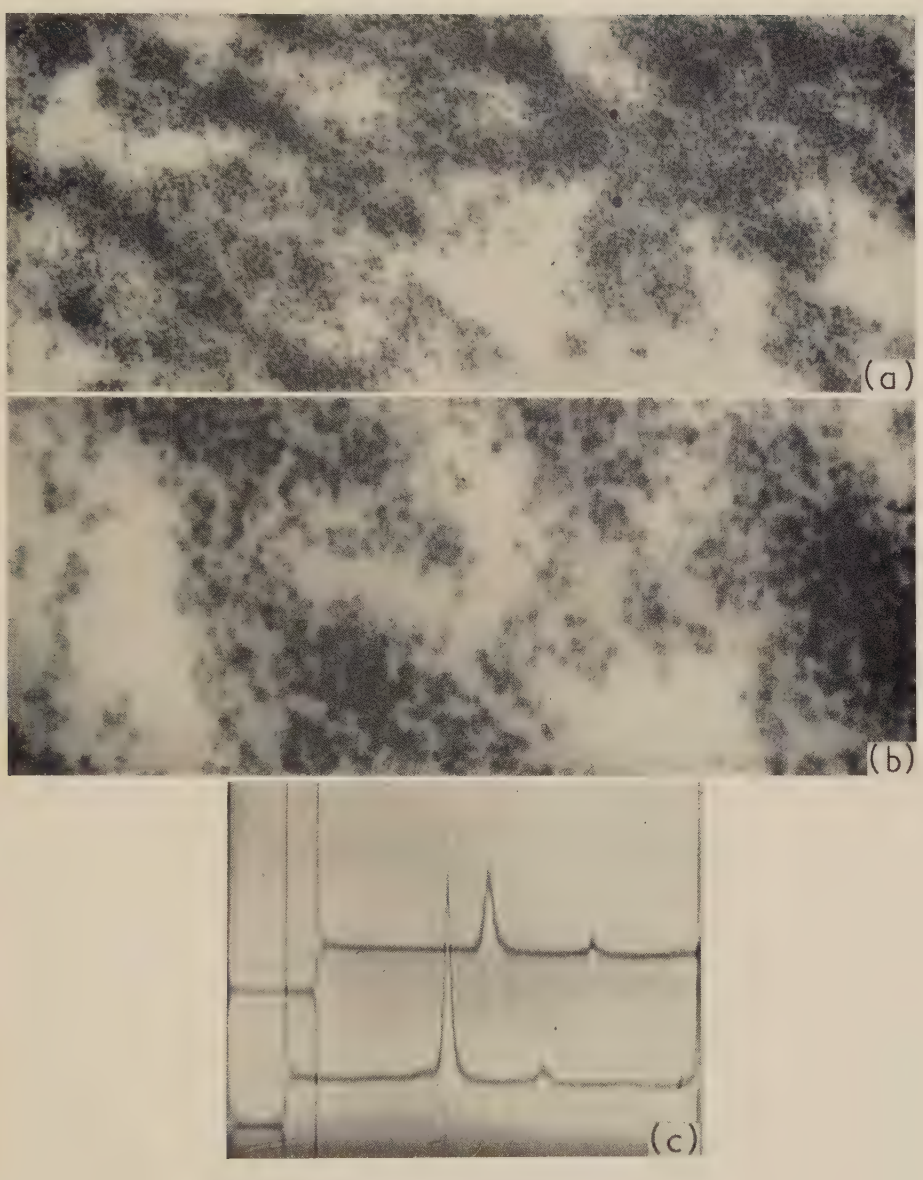

PLATE I. Electron microscope and ultracentrifuge analysis of ribosomes.

(a) Low magnification view of a portion of a pellet of DC-2 ribosomes. Magnification × 33,000.
(b) Higher magnification view of ribosomes shown in (a). Magnification × 92,000.
(c) Schlieren patterns of two preparations of DC-2 ribosomes centrifuged 10 min at 35,600 rev/min. Upper pattern from ribosomes stored 1 week at 0°C, lower pattern from freshly prepared ribosomes. Sedimentation from left to right.

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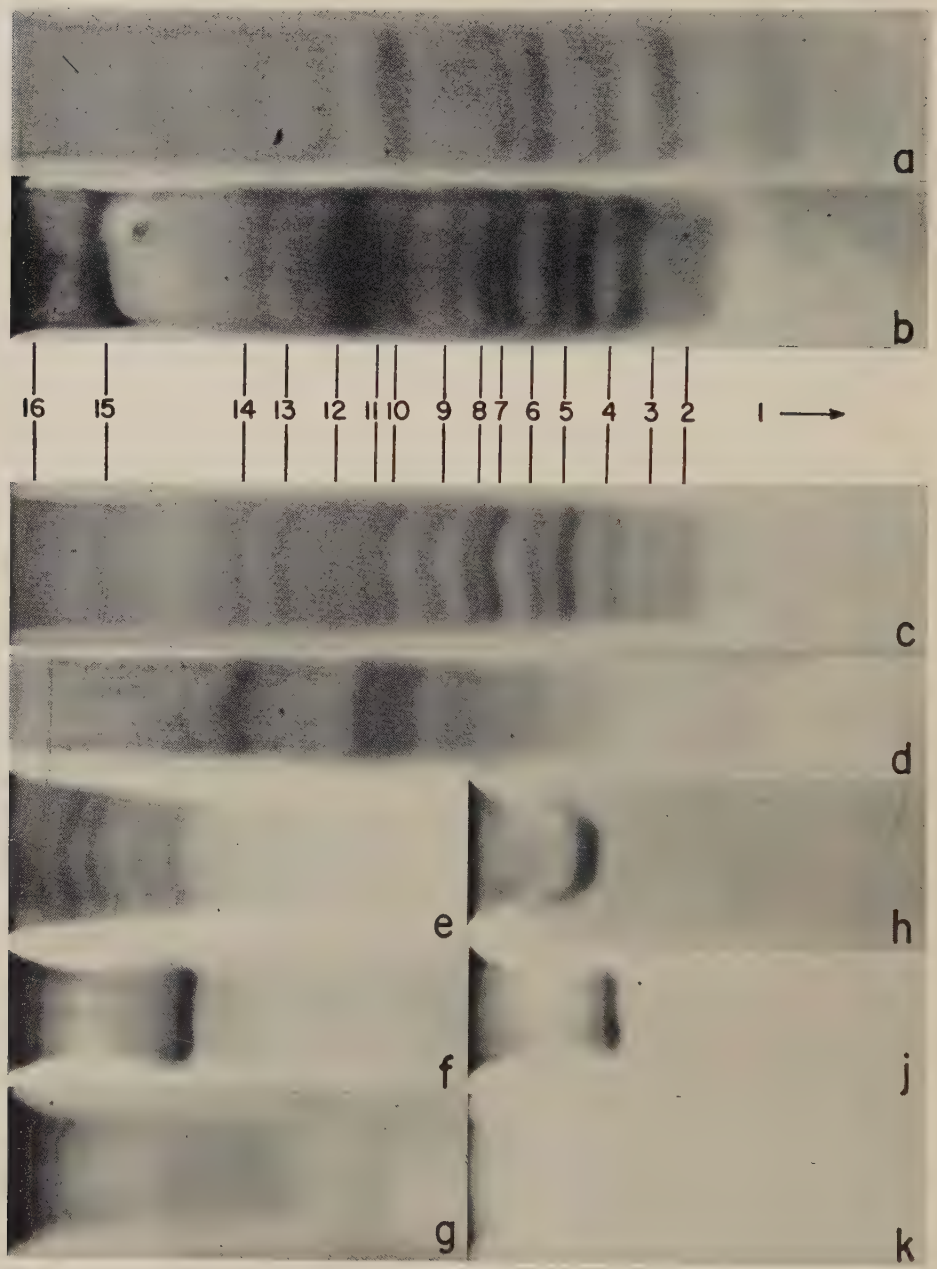

PLATE II. Electrophoretic patterns of acid-soluble ribosomal proteins in starch gel. (b) and (c) were from separate preparations of ribosomes. Migration from left to right.

(a) pH 3·3, formate buffer. Nigrosin.
(b) pH 4·2, acetate buffer. Nigrosin.
(c) pH 4·2, acetate buffer. Amido black.
(d) pH 5·2, acetate buffer. Nigrosin.
(e) pH 6·1, cacodylate buffer. Amido black.
(f) pH 7·1, veronal buffer. Nigrosin.
(g) pH 7·9, borate buffer. Amido black.
(h) pH 8·5, tris buffer. Nigrosin.
(j) pH 8·6, glycine buffer. Nigrosin.
(k) pH 8·6, glycine buffer. Amido black.

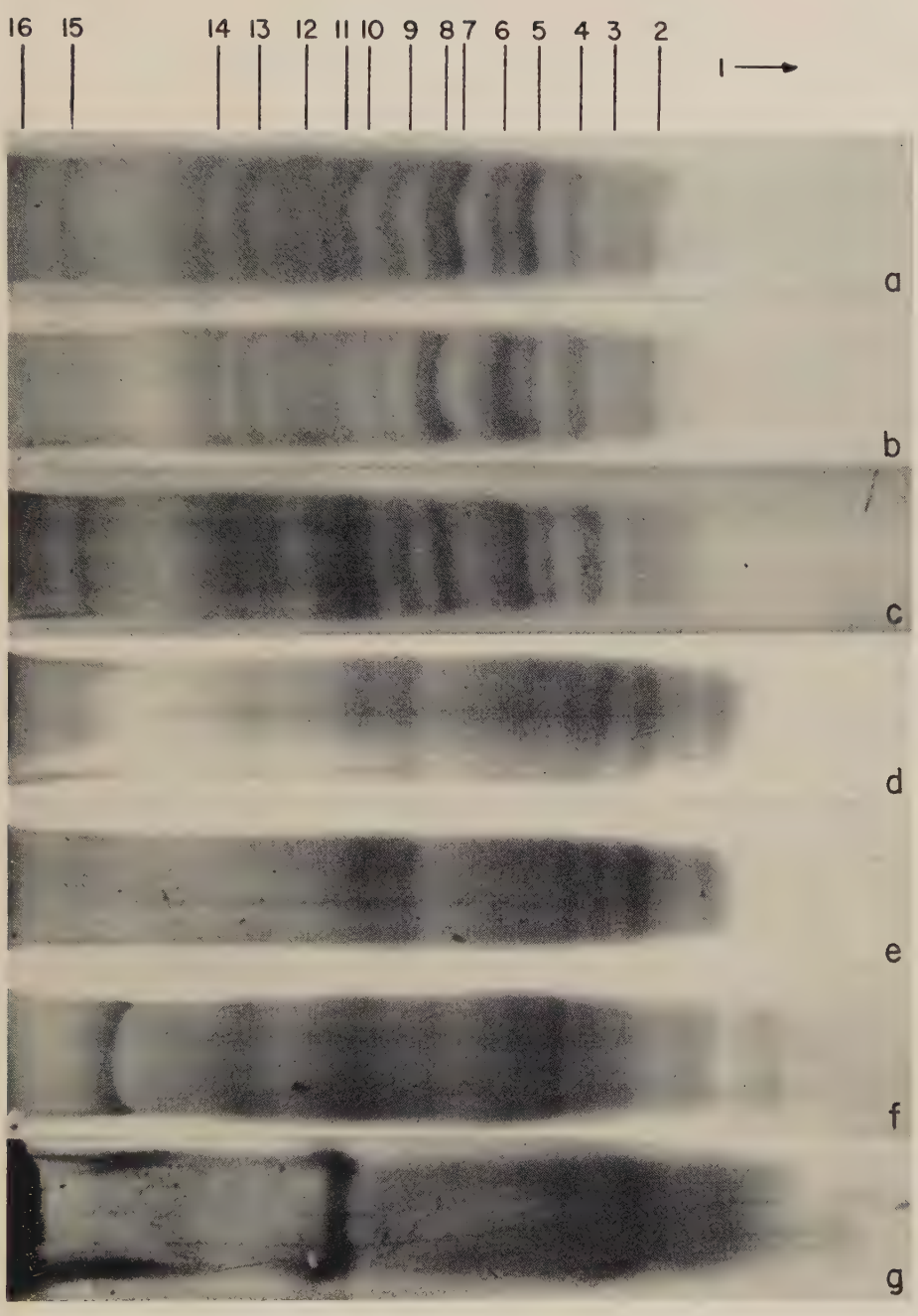

PLATE III. Electrophoretic patterns of acid-soluble ribosomal proteins and histones in starch gel. All at pH 4·2, acetate buffer. Migration from left to right.

(a) Untreated proteins. Same as Plate II(c). Amido black.
(b) Proteins from ribosomes treated with 0·3% DOC at the DC-1 level. Amido black.
(c) Proteins dialysed overnight at 0°C against 0·02 M-acetate buffer, pH 4·2. Amido black.
(d) Untreated proteins using 8 mm slot in gel. Nigrosin.
(e) Proteins treated with 4 M-urea and dialysed overnight at 0°C against 0·02 M-acetate buffer, pH 4·2. 8 mm slot. Nigrosin.
(f) Proteins treated with 4 M-urea and run in a gel containing 4 M-urea. 8 mm slot. Nigrosin.
(g) Presumed histones obtained from acid extraction of nuclear fraction. Nigrosin.

deoxycholate during fractionation produced only minor changes in the electrophoretic patterns given by the acid extract. For example, comparing Plate III(a) and (b) the only important changes are the apparent absence of zones 10 and 15 in the DOC-treated material. Concentration of the ribosomal proteins by pervaporation at 4 °C had no significant influence on the pattern except to increase the concentration of all components. Storage of the protein extract at 0° to 5 °C at pH 3·7 for up to 10 days did not cause any change in the pattern. Dialysis of the extract against pH 4·3 acetate buffer for 12 hr at 5 °C caused minor changes in the region of zones 6 to 8, for reasons unknown, and removed zone 1, but the general electrophoretic pattern remained the same (cf. Plate III(a) and (c)).

To examine the possibility that protein complexes dependent on secondary forces may have contributed to the apparent heterogeneity the protein extract was made 4 M with respect to urea. In one experiment the urea solution was dialysed against 0·02 M-acetate buffer (pH 4·3) and then subjected to electrophoresis in an ordinary gel while in another experiment the urea solution was run directly in a gel made up in 4 M-urea. The former treatment caused no change in the electrophoretic pattern (Plate III(d) and (e)) while the run in urea gel showed only minor changes such as an extra zone at the front of the pattern and an intense zone in the region of zone 15 in the single slot patterns (Plate III(d) and (f)). The mobility of the proteins was slightly less in the urea gel due to the lower voltage gradient.

Crude histone extracted from the nuclear fraction (Ts'o & Sato, 1959) was also subjected to electrophoresis at pH 4·2 (Plate III(g)). The pattern, quite distinct from that of the ribosomal proteins, showed a few fast moving zones and a sharp slow zone. The slow zone stained strongly with nigrosin but only very weakly with amido black. The histone pattern could not be intensified by pervaporation because, unlike the ribosomal proteins, the histone aggregated during this process. This aggregation has also been noted with animal histones (Neelin & Butler, 1959).

(c) *Analytical studies*

Table 1 compares N-terminal amino acids of the intact ribosomes, the acid-soluble ribosomal proteins and the residue remaining after acid extraction of the ribosomes. Two methods of hydrolysis, refluxing (24 hr in 5·5 M-HCl) and sealed bulb (4·5 hr in conc. HCl at 100 °C), were applied to the intact ribosomes. The milder sealed bulb method gave greater recoveries of DNP-glycine, lysine, and serine, but reduced yields of other residues, probably because of their participation in relatively resistant peptide linkages. The intact ribosomes contained 10 significant N-terminal residues, if the yields from both methods are considered. Neither DNP-proline nor its derivatives were observed, even after sealed bulb hydrolysis. The ribosomal protein was hydrolysed by the sealed bulb method and no new end groups were observed. The proportion of N-terminal glutamic acid, aspartic acid, and serine was reduced in the extracted proteins giving a corresponding increased proportion of the other residues. The general similarity of the end groups of the extracted protein and the intact ribosomes is further illustrated by the results from reflux hydrolysis of the intact ribosomes and the acid-insoluble residue. Here the end groups of the acid-soluble ribosomal protein were calculated as the difference between the recovered DNP-amino acids of the whole ribosomes and of the residue. The DNP residues, recovered after reflux hydrolysis of the intact ribosomes, were estimated to total about 4 μmole/g ribosome. Because of the methods of hydrolysis used and the presence of large amounts of RNA in some of the samples meaningful molecular weights could not be calculated.

Nitrogen and phosphorus values were determined on several preparations of ribosomes and the results are shown in Table 2. The N/P ratios of the ribosomes showed some variation but in general were consistent with the chemical composition of ribosomes of pea seedlings reported by Ts'o *et al.* (1956). Nitrogen and phosphorus determinations on the acid-soluble protein consistently showed that this fraction

TABLE 1

Relative proportions of N-terminal amino acids of pea bud ribosomes and acid-soluble ribosomal proteins

Amino acid	% of total recovered DNP-amino acids				
	Ribosomes (sealed)	Acid-soluble ribosomal proteins (sealed)	Ribosomes (reflux)	Residue (reflux)	Acid-soluble ribosomal proteins (calculated)
Valine	10	26†	28	28	28
Leucine	10		7	3	12
Alanine	13	19	29	24	32
Glycine	18	29	7	7	6
Lysine	12	16	1	trace	3
Threonine	trace	trace	7	8	6
Glutamic acid	9	10†	3	3	3
Aspartic acid	11		6	7	5
Serine	17		6	11	1
Methionine	0	0	6	9	4
Histidine	trace	0	trace	trace	—
Phenylalanine	trace	0	trace	trace	—

† These residues were not well separated on the chromatogram and were analysed together.

TABLE 2

Results of nitrogen and phosphorus analyses of ribosomes and acid-soluble ribosomal proteins

Experiment	Ribosomal fraction †	Nitrogen (mg)			Phosphorus (mg)			N/P for ribosomes
		Ribosomes	Acid-soluble proteins	% Recovery in acid-soluble proteins	Ribosomes	Acid-soluble proteins	% Recovery in acid-soluble proteins	
1	DC-3	53·7	9·3	17·3	16·4	0·24	1·5	3·3
2	DC-2	27·4	4·6	16·8	4·8	0·18	3·8	5·7
3	DC-2	85·7	15·5	18·1	17·5	0·28	1·6	4·9
4	DC-2	57·4	10·1	17·6	14·5	0·24	1·7	4·0
Mean	—	—	—	17·5	—	—	2·2	4·5

† See Materials and Methods.

contained about 17·5% of the nitrogen and 2% of the phosphorus of the ribosomes. Dialysis of the acid extract removed the phosphorus.

Ultraviolet spectral analyses (Fig. 1) paralleled the studies of nitrogen and phosphorus. The absorption spectrum of the intact ribosomes showed a broad peak with a maximum at 2550 Å, as reported by Ts'o *et al.* (1956) while the spectra of the acid extracts consistently showed a peak in the region of 2650 Å to 2700 Å. Dialysis of the extract considerably reduced the optical density and the maximum shifted to 2750 Å.

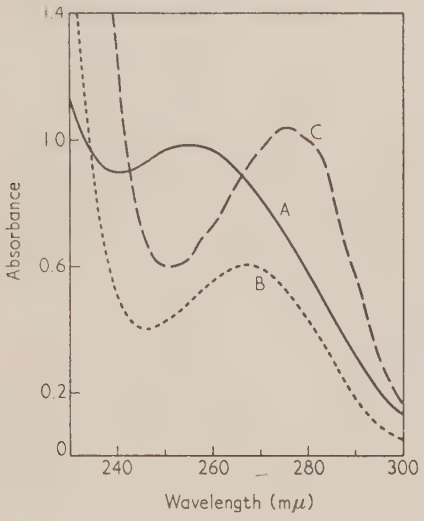

Fig. 1. Ultraviolet absorption spectra of ribosomes, and of acid-soluble ribosomal proteins before and after dialysis.
A. Dilute suspension of intact ribosomes.
B. Acid extract of ribosomes (1 volume of extract diluted with 3 volumes of 0·02 M-acetate buffer, pH 4·2).
C. Same acid extract as in B, dialysed overnight against 0·02 M-acetate buffer, pH 4·2 (undiluted).

The anthrone test for carbohydrate applied to HCl extracts was positive before and negative after dialysis.

Taken together the above analytical results indicate that the acid extract contained about 17·5% of the nitrogen of the ribosomes and a small amount of contaminating nucleotide material which can be removed by dialysis. The 17·5% nitrogen may be considered to be derived entirely from ribosomal protein and represents approximately 29% of the protein of the intact particles, assuming that the particles contain 55% protein and 35% RNA (Ts'o *et al.*, 1956) and that the percentage nitrogen in RNA is the same as in protein.

(d) *Ultracentrifuge analyses of the acid soluble ribosomal proteins*

The sedimentation pattern of ribosomal proteins showed a main broad peak of sedimentation coefficient about 0·9 s and a much smaller, more rapidly sedimenting peak which soon diffused (Fig. 2). The results obtained by the Archibald method are plotted in Fig. 3, and from the slope of the line the molecular weight was estimated as 18,000. The usefulness of this result is limited by the evident polydispersity of the proteins and particularly by the presence of a separate heavier component. However, it serves to establish the order of magnitude of the molecular weights.

4. Discussion

The foregoing results show that with mild hydrochloric acid treatment about 29% of the protein of ribosomes from pea buds is extracted and that the extracted protein

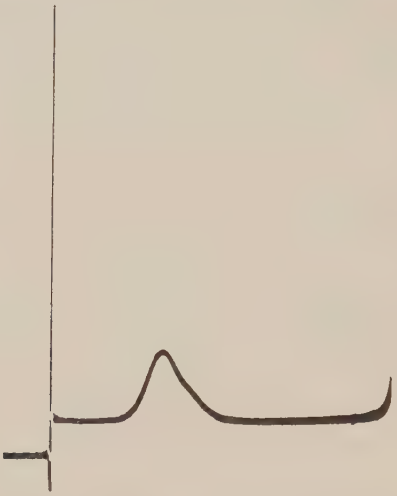

FIG. 2. Schlieren pattern from ultracentrifugation of acid-soluble ribosomal proteins 4 min at 59,780 rev/min in a synthetic boundary cell. A small amount of a more rapidly sedimenting component appears as a shoulder on the right of the main peak. Sedimentation from left to right.

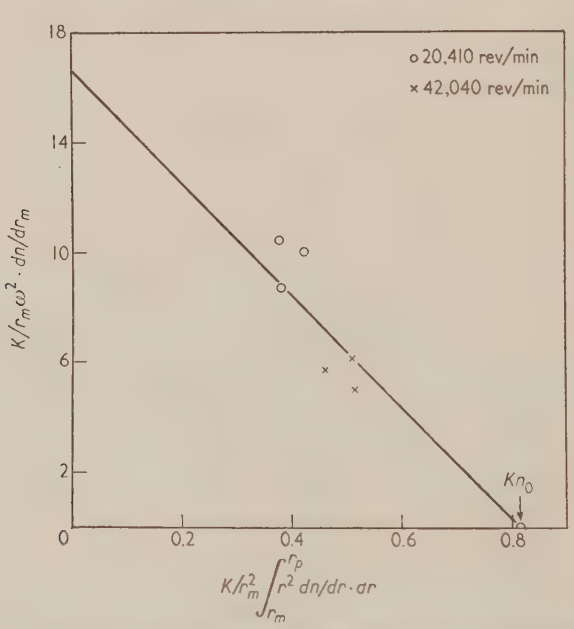

FIG. 3. Graph of measurements obtained from Archibald sedimentation analysis of acid-soluble ribosomal proteins.

components give a complex electrophoretic pattern in starch gel, with at least 16 components resolved at pH 4·2. Relative intensities and sizes of the electrophoretic zones are markedly different and the overall electrophoretic pattern is complicated and

distinctive. The pattern is quite reproducible in separate preparations from similar tissue and the components of the pattern are stable on storage. The isoelectric points of the proteins lie around pH 8 and the average molecular weight is about 18,000. Valine, leucine, glycine, alanine, and lysine are the main N-terminal amino acid residues present. N-terminal proline is absent, indicating that the proteins are qualitatively different from an acid-soluble protein fraction of rat liver microsomes recently described by Butler, Cohn & Simson (1960).

Several studies were performed to rule out possible sources of error. Ultracentrifuge and electron microscope analyses of the microsomal fraction showed it to consist almost entirely of ribosomes. In addition, treatment of the crude microsomal fraction with deoxycholate to disperse possible membranous components did not affect the electrophoretic behaviour of the basic proteins extracted from the purified microsomal fraction. An extra cycle of purification of the microsomal fraction did not influence the electrophoretic results, indicating that contribution to the results by other centrifugal fractions was insignificant. Histone, a possible contaminant, gave a distinctly different electrophoretic pattern from that obtained with the ribosomal proteins. The acid-soluble ribosomal proteins further differed from the typical behaviour of histones (Neelin & Butler, 1959) in remaining stable during pervaporation and not aggregating above pH 5 in the absence of urea. Since the patterns were essentially unchanged by treatment with 4 M-urea, both before and during electrophoresis, hydrogen-bonded protein complexes do not appear to be responsible for the complex elcctrophoretic behaviour. The possibility that nucleic acids bound to proteins made significant contribution to the electrophoresis results was eliminated by phosphorus and spectral analyses following dialysis. Determinations of N-terminal amino acid residues indicated that the relatively mild acid extraction did not generate any appreciable amount of new end groups and therefore probably caused little or no significant degradation of the native proteins of the ribosomes. Taken together, these control studies support the interpretation that the distinctive complex of proteins revealed by starch gel electrophoresis represents a real group of basic proteins present in intact ribosomes of pea buds.

Since only about 29% of the protein of the ribosomes is removed by cold HCl at pH 1·7, the nature of the residual protein is of interest. It is possible that the relatively mild HCl treatment removed only a portion of each of the proteins present in the particles. The general similarity of the N-terminal amino acids of the intact ribosomes and the acid-soluble proteins would support this interpretation. On the other hand, the acid-soluble proteins may represent a group of proteins which are quite distinct from those insoluble in acid. This possibility is suggested by the finding of Butler *et al.* (1960) that acid-soluble proteins of rat liver microsomes are considerably richer in basic amino acids than the proteins of the whole microsomal fraction. The existence of two distinct groups of proteins is also indicated by the fact that further treatment of the acid-insoluble residue with HCl did not extract a significant amount of protein.

The biological significance of the acid-soluble ribosomal proteins is unknown at this stage. However, the results presented allow several inferences to be drawn. It is obvious that the protein composition of pea bud ribosomes is extremely complicated even if the majority of all proteins in the ribosomes are qualitatively represented in the hydrochloric acid extract. Such complexity has also been inferred by Yin & Bock (1960) for ribosomes of yeast. The large number of relatively small proteins could all be present in the same particle, giving structures of great intricacy, or on the other

hand, the complex of proteins may reflect the presence of a number of chemically distinct particles in the fraction. In this latter situation the proteins may confer specificity on the RNA of the particles in a manner analogous to that suggested for histone and DNA (Stedman & Stedman, 1950). It is also possible that some of the protein components represent substances being synthesized by the ribosomes and that some are responsible for specific antigenic responses of the type reported for microsomes of oats (Wright, 1960).

Finally, it might be noted that the presence in ribosomes of considerable amounts of protein soluble in dilute mineral acid could seriously interfere with attempts to isolate histone proteins from whole cells. This factor probably accounts in part for the finding of large amounts of protein soluble in 0·2 M-HCl in ribosome rich cells of pea embryos (Stern, Johnston & Setterfield, 1959). It is also probable that the acid-soluble ribosomal proteins, with their relatively high isoelectric points are responsible for the mild cytoplasmic reaction of meristematic plant cells in fast green at pH 8.

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Preparation and Characterization of Artificial Top Component from Turnip Yellow Mosaic Virus

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Purified preparations of turnip yellow mosaic virus devoid of natural top component release their ribonucleic acid on treatment with a buffer of pH 12 at 30°C. This process takes place very rapidly and leaves, in addition to the ribonucleic acid, empty protein shells and protein fragments in the reaction mixture.

The protein shells can be isolated by a simple differential centrifugation procedure. This shell material has been characterized by its ultraviolet absorption spectrum, by sedimentation analysis, by diffusion, electrophoresis, electron microscopy, crystallization and X-ray diffraction, and has thus been shown to be identical with natural top component.

1. Introduction

Turnip yellow mosaic virus (TYMV) belongs to the group of so-called "spherical plant viruses", and is one of the members of this group which have been well studied. It is fairly well established that the virus consists of a protein shell, probably polyhedral in shape, and is composed of smaller subunits (Klug, Finch & Franklin, 1957*a*,*b*; Nixon & Gibbs, 1960; Huxley & Zubay, 1960; Klug & Finch, 1960). Within this shell is RNA, which accounts for 37% of the total mass (Markham, 1959). Under the electron microscope the outward appearance of the virus is spherical (Cosentino, Paigen & Steere, 1956) or, sometimes, polyhedral (Cosslett, 1956), and some evidence for the existence of surface structure of the particles has been described (Steere, 1957).

In the diseased plant two types of particles are found to be associated with the infection. One type, ribonucleoprotein, is infectious, while the other is purely protein in character and is non-infectious (Markham & Smith, 1949). These particles can be separated and purified to a considerable degree (Markham, 1951; Cosentino *et al.*, 1956). Physico-chemical and chemical comparison of the properties of the virus and its so-called "top component" indicated that the particles were identical, except that the latter lacked RNA (Markham, 1951; Cosentino *et al.*, 1956; Fraser & Cosentino, 1957).

The role of the empty top component in the infection process has been the subject of much experimentation and speculation (Markham, 1953; Jeener, 1954; Matthews, 1958, 1959), but so far no completely satisfactory answer has been obtained.

Since the recognition of RNA as the infectious component in tobacco mosaic virus (TMV) (Gierer & Schramm, 1956; Fraenkel-Conrat, Singer & Williams, 1957), much effort has been devoted to demonstrating this property as a general principle in other purified viruses (Kaper & Steere, 1959*a*,*b*; Rushizky & Knight, 1959; Koch, Koenig & Alexander, 1960). Relatively few attempts have been made to isolate the protein components of these viruses in a native state (Sinclair, Geil & Kaesberg, 1957; Lyttleton & Matthews, 1958; Kaper & Steere, 1959*b*) despite the important role they

might be expected to perform in reconstitution studies, and in studies on virus structure and synthesis.

The present publication describes what is apparently the first successful attempt to prepare and characterize the protein from TYMV in an undenatured state. A preliminary account of some of this work has already been published (Kaper, 1960).

2. Materials and Methods

Virus preparations. TYMV was grown in the Wong Bok variety of Chinese cabbage (*Brassica chinensis L.*) under greenhouse conditions. The virus was purified by means of Steere's (1956) butanol-chloroform procedure, by which virus preparations can be obtained free from accompanying protein components, including top component (Cosentino *et al.*, 1956). Plate I(a) shows the sedimentation diagram of the virus purified by this method. Virus prepared in this way was always used as starting material for the preparation of the virus protein (artificial top component).

The virus used as a standard of comparison (Plate I(c)) for the "reconstructed" TYMV + artificial top component system, was a top component-rich preparation made by a differential centrifugation procedure, and was a gift from Dr. J. T. Finch and Dr. A. Klug of Birkbeck College Crystallography Laboratory, University of London.

Ultracentrifugation. All the preparative work involved in the isolation of virus and in the preparation of artificial top component was performed in the Spinco model L ultracentrifuge.

The Spinco model E ultracentrifuge equipped with schlieren optics was used for ultracentrifugal analysis. Unless otherwise stated, the samples were suspended in a buffer of pH 7, ionic strength 0·1 (Miller & Golder, 1950). All experiments were conducted at 20°C.

Diffusion. Diffusion experiments were performed in the Spinco model E ultracentrifuge operated at low speed, using the valve type synthetic boundary cell. Artificial top component was suspended in the pH 7 buffer (ionic strength 0·1). The temperature used was 20°C. Diffusion coefficients were calculated from measurements of the areas and of the maximum ordinates under the schlieren gradient curves.

Electrophoresis. A Kern LK 30 micro-electrophoresis apparatus was used, and the boundaries were observed by a Jamin interference optical system. Since this apparatus is not particularly suited to the precise measurement of electrophoretic mobility, preparations (of artificial top component and virus) for mobility comparison were mixed in equal concentration, and dialysed repeatedly against buffer. Electrophoresis was then performed at room temperature.

Ultraviolet spectra. Ultraviolet spectral and optical density measurements were made with a Beckman DK2 automatic recording spectrophotometer and a Unicam SP500 spectrophotometer, respectively.

Photographs of crystals. A Leitz Ortholux microscope with a camera attachment was used to photograph crystals of artificial top component. Photographs were taken at a magnification of 400×, with either phase contrast or dark field illumination. The crystals were suspended in their mother liquor (0·9 to 1·0 M-ammonium sulphate).

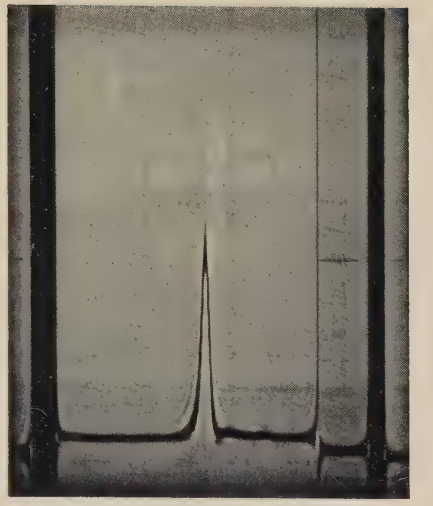
Electron microscopy. Electron micrographs of the particles of artificial top component were kindly made by Dr. R. L. Steere, Plant Virology Laboratory, U.S. Dept of Agriculture, Beltsville, Maryland, U.S.A. The particles were either air-dried from a suspension in 2% ammonium acetate, or frozen dried from a distilled water suspension.

Plate I(a). Sedimentation diagram of TYMV used as starting material, showing absence of natural top component. Virus concentration: 3 mg/ml. Picture taken 21 min after attaining speed of 23,150 rev/min.

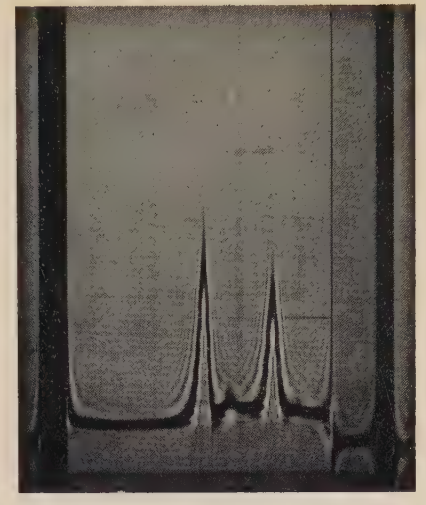
(b) Sedimentation of artificial top component. Concentration: 2·4 mg/ml. Pictures taken at 4 min intervals with the first photograph 5 min after attaining speed of 33,450 rev/min.

(c) Sedimentation diagram of "top component-rich" TYMV. Picture taken 23 min after attaining speed of 23,150 rev/min.

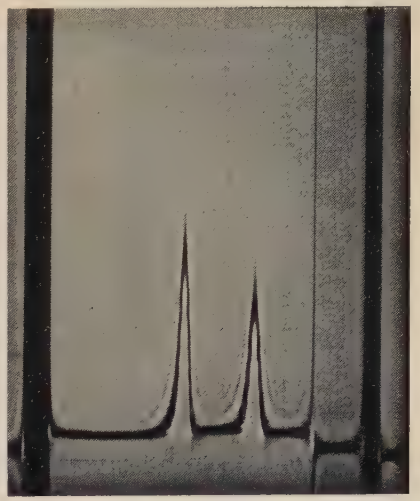
(d) Sedimentation diagram of "reconstructed" TYMV + artificial top component. Concentrations, TYMV, 2·2 mg/ml.; artificial top component, 1·25 mg/ml. Picture taken 23 min after attaining speed of 23,150 rev/min.



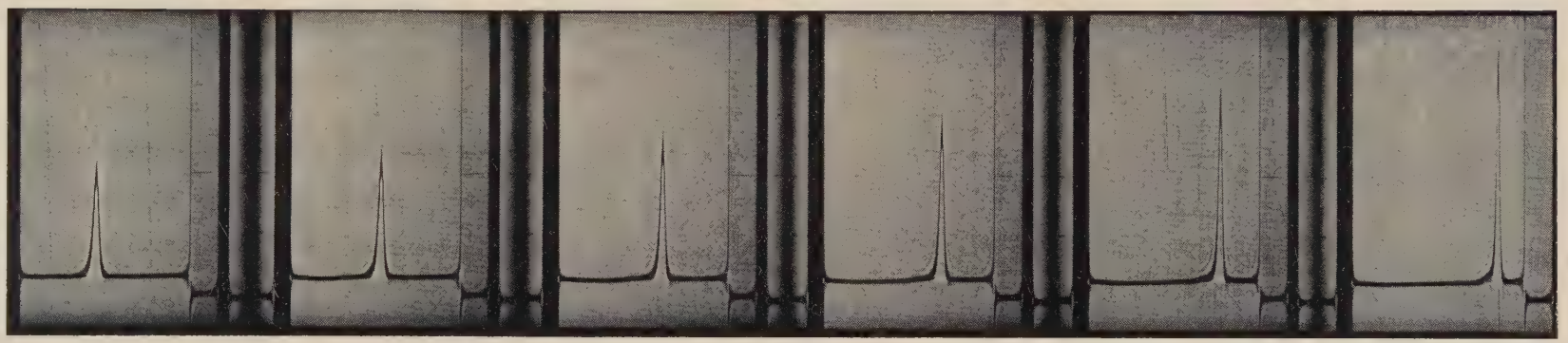
(a)



(c)



(d)



(b)

PLATE I. (For legend see facing page.)

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X-ray diffraction. Powder diagrams of small crystals, obtained by crystallization from ammonium sulphate solution, were made by Dr. J. T. Finch and Dr. A. Klug, Birkbeck College Crystallography Laboratory, University of London (see following paper, Finch & Klug, 1960).

Protein concentration. Protein concentrations were determined by the Folin phenol method (Lowry, Rosebrough, Farr & Randall, 1951) and were based on a standard curve using whole virus of known concentration corrected for an RNA content of 37%. The results obtained by this method agreed with those based upon micro-Kjeldahl nitrogen analysis, and with those calculated from analytical ultracentrifuge experiments, using a value for the specific refractive increment of protein = 0·00186.

3. Experimental Results

(a) *Preparation of artificial top component*

In previous communications on this subject (Kaper & Steere, 1959*b*; Kaper, 1960) the use of prolonged ($2\frac{1}{2}$ days) alkaline treatment for the degradation of TYMV was described. These efforts were aimed at obtaining small protein fragments of the virus with a view to their reaggregation—with or without RNA—to reconstituted virus, or to an artificial top component. Such reaggregation had already been shown for TMV (Fraenkel-Conrat & Williams, 1955). However, the results of experiments performed at different degrees of alkalinity (Kaper, 1960) suggested that RNA from TYMV could escape from the virus particle while leaving the protein shell intact, and that more of the smaller protein fragments appeared at the higher pH values.

Recent studies in this laboratory on the sequence of events during this treatment have fully confirmed this view and will be published in due course. Our picture of the first stages of the reaction at pH 12 is now quite clear. Under the reaction conditions to be described, the following events happen. Within a few minutes of being adjusted to pH 12, the virus suspension becomes turbid: this turbidity results from a precipitate formed from relatively large amounts of cations (Ca^{2+} and Mg^{2+}) released from the virus, and probably phosphate ions from the reaction mixture. (The metal ions were detected by paper chromatography of a solution of the precipitate in dilute HCl. This solution failed to give any characteristic u.v. absorption spectrum. Its inorganic nature became clear on ashing.) If the reaction is stopped at this stage, and the mixture analysed, the virus is found to have become degraded almost completely into relatively large RNA fragments (4 S to 8 S), empty protein shells, and protein fragments of varying size. Further treatment removes all traces of intact virus, hydrolyses the RNA to smaller fragments, and forms larger amounts of smaller protein material at the expense of the complete shells and larger protein fragments. From these observations a standard preparation procedure for artificial top component has been devised.

Purified virus in suspension is mixed with a pH 12 K_2HPO_4—NaOH buffer to give a final virus concentration of 1%, and a buffer strength of 0·14 M (these figures are to be regarded to some extent as approximate; they derive from expediency in the use of available material. In this laboratory, for example, it proved convenient to mix 1 part of a 3·4% virus suspension in 0·01 M, pH 7 phosphate buffer with 2·4 parts of a 0.2 M, pH 12 phosphate buffer).

The reaction mixture is incubated at 30°C for about 30 min, followed by low-speed centrifugation to remove the inorganic precipitate. The opalescent supernatant is then centrifuged for 2 hr at 40,000 rev/min (no. 40 rotor). The pellet resulting from this treatment is resuspended in the standard pH 7 buffer (ionic strength 0·1) and is then

subjected to a further two cycles of ultracentrifugation and washing. The supernatant solutions from the ultracentrifuged preparations may be discarded.

Ultracentrifugation in water cannot be advised, as it was found to cause a loss of protein, probably as a result of charge effects. The properties of the resulting material, described hereafter as "54 s component" or as "artificial top component", are given in the subsequent section of this paper.

Small quantities of 54 s component have been prepared under different reaction conditions from those cited above (shorter treatment, lower pH, different temperature); such preparations were identical with the standard preparation, but involved slight changes in isolation procedure.

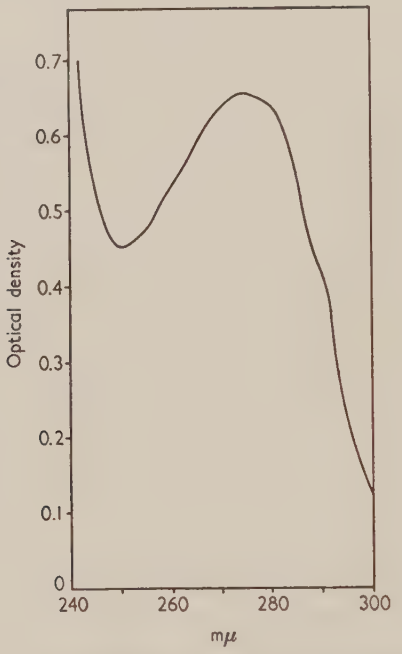

Fig. 1. Ultraviolet spectrum of artificial top component. Concentration: 0·48 mg/ml. in ionic strength 0·1 pH 7 buffer.

(b) *Identification of artificial top component with natural top component*

For purposes of identification, comparisons between natural top component and artificial top component have been restricted to a range of physico-chemical properties. Wherever possible, these properties were evaluated independently and the absolute values for the two systems compared. As has been mentioned previously, in the case of electrophoresis, the properties of artificial top component were compared with those of natural top component in a mixed system.

(i) *Ultraviolet system*

Allowing for a considerable degree of light scattering due to particle size, the u.v. spectrum of artificial top component is that typical of a protein (Fig. 1). A solution of artificial top component in pH 7 buffer (ionic strength 0·1) at a concentration of 0·5 mg/ml. has an extinction value of 0·68 at 275 mμ. This figure compares favourably with Cosentino's (Cosentino *et al.*, 1956) value of 0·70 at 280 mμ for natural top component at the same concentration.

(ii) *Sedimentation, diffusion and molecular weight*

Artificial top component sediments as a sharp and symmetrical boundary (Plate I(b)). The sedimentation coefficient was measured over a range of concentrations in pH 7 buffer (ionic strength 0·1). The values obtained were corrected on a water basis, and extrapolated to zero protein concentration (Fig. 2). A value of 54·5 ± 0·5 Svedberg units was obtained for artificial top component.

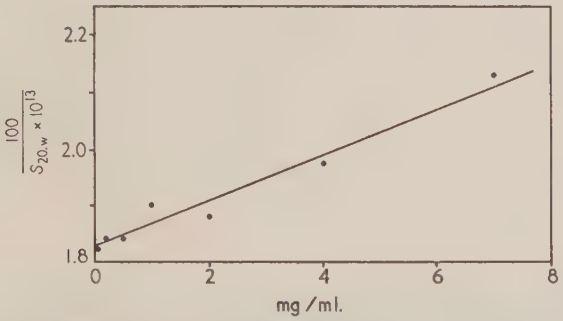

Fig. 2. Concentration dependence of sedimentation coefficient of artificial top component.

This value of 54·5 s differs significantly from the figure of 48·8 s for natural top component quoted by Markham (1951). A number of experiments was therefore performed comparing a system of "natural" virus + top component with a system of "reconstructed" virus + artificial top component. In a previous communication (Kaper, 1960) such an experiment was described. The "natural" mixture used for that experiment was rather poor in top component, and to remove all doubt, a top component-rich preparation—isolated by Dr. Finch—was used on this occasion. This was compared with another "reconstructed" system, and the results are shown in Plates I(c) and I(d). The values of $S_{20,w}$ obtained for the "natural" system were 110·5 s and 53 s, for virus and top component respectively. In the case of the "reconstructed" system, 112 s was found for the virus, and 54 s for the artificial top component.

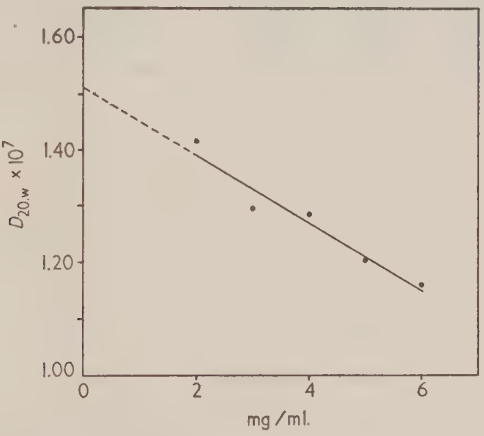

Fig. 3. Extrapolation of diffusion coefficients of artificial top component to zero protein concentration.

Diffusion experiments were performed at five different concentrations of artificial top component (Fig. 3). The apparent diffusion coefficients obtained from the

height-area measurements were plotted against $1/t$, and extrapolated to infinite time to find the corrected values for D. Zero time corrections calculated from these plots did not exceed 12 min. After correction on a water basis, the individual values were plotted against protein concentration, and by linear extrapolation a figure of $1{\cdot}51 \pm 0{\cdot}04 \times 10^{-7}$ cm^2/sec was obtained for $D_{20,w}$ (Fig. 3).

Using this value for the diffusion coefficient, and 54·5 s for the sedimentation coefficient, and assuming a partial specific volume of 0·75, the molecular weight of artificial top component, calculated by means of the Svedberg equation, was found to be 3·5 million. The difference between this and Markham's value (1951) of 3 million for natural top component results largely from the higher sedimentation coefficient found in this work. It is, however, in excellent agreement with the upward revision for the virus particle weight mentioned by Klug & Finch (1960) as a private communication from Dr. R. Markham, and with their proposal of 180 chemical units of approximately 20,000 molecular weight in TYMV.

(iii) *Electrophoresis*

During electrophoresis, artificial top component migrates as a single boundary towards the anode. Its electrophoretic mobility is identical with that of the virus over the pH range investigated (pH 4·1 to 9·5). Plate II is a representative picture of an electrophoresis run performed with a mixture of virus + artificial top component.

(iv) *Electron microscopy*

Electron micrographs (Plate III(a) and III(b)) of artificial top component, made by Dr. Steere, have also proved beyond doubt that this material is very similar to natural top component. As with natural component (Cosentino *et al.*, 1956), the particles dimple or even collapse upon air drying, which shows that they are hollow, while freeze drying preserves their structural integrity. In the latter picture, the observed diameter fits in with the ones measured for natural top component.

(v) *Crystallization and X-ray diffraction*

Artificial top component crystallizes with the same ease as is described for TYMV and natural top component (Markham & Smith, 1949). Adding one half volume of saturated ammonium sulphate to a suspension of artificial top component (concentration approximately 5 mg/ml.) causes the formation of a micro-crystalline precipitate. Larger crystals may be obtained by making the suspension of artificial top component only 26 to 27% saturated with ammonium sulphate (procedure worked out by Dr. Klug and Dr. Finch for growing larger crystals). After a few days at room temperature, nicely formed typical crystals may be observed (Plates IV(a) and (IVb)).

Dr. Finch and Dr. Klug (see following paper, Finch & Klug, 1960) have made powder photographs from a sludge of these crystals, and found them to be identical with corresponding pictures of natural top component. Low-angle photographs showed the same crystal diffraction lines for the two types of top component, corresponding to an identical crystal structure. The particle scattering was as for that of a hollow sphere with the same dimensions as natural top component. In high-angle photographs, at a resolution of 10 Å, the two types of top component appeared identical, but small differences would not show in an unoriented photograph.

However, these results also point to the essential identity of the natural and the artificial top components.

PLATE II. Electrophoresis pattern of a mixture of TYMV + artificial top component in equal amounts. Total concentration of material: 3·5 mg/ml. Buffer: ionic strength 0·1, pH 7.

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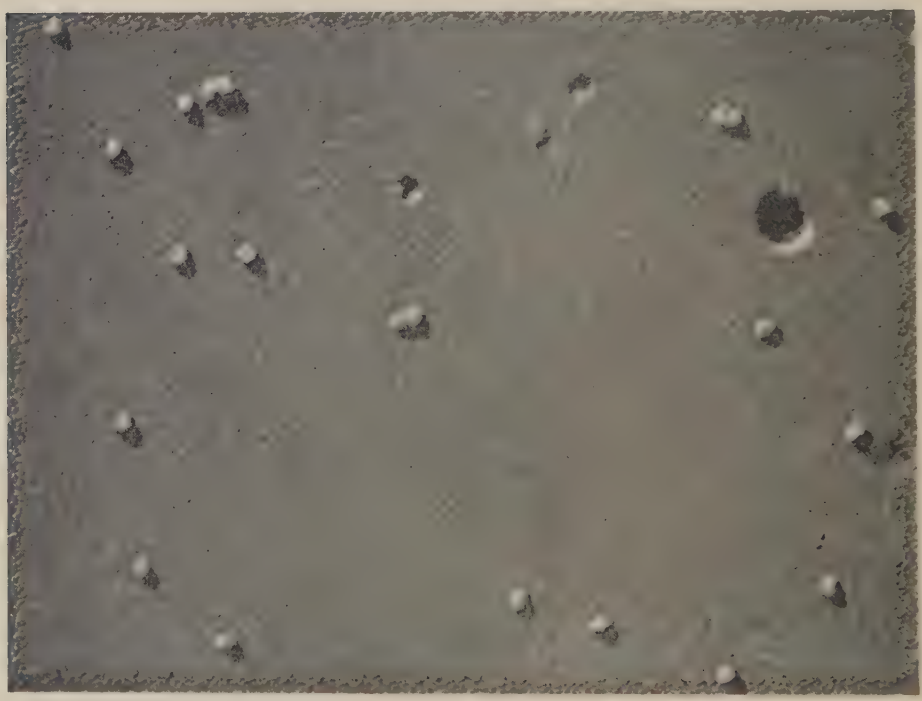
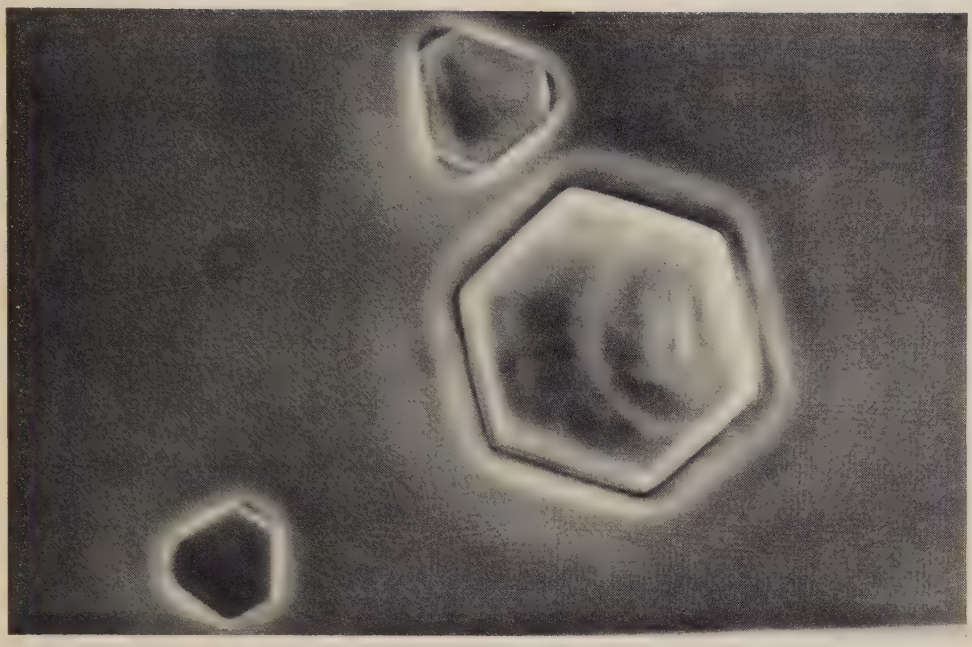
(a)



(b)

PLATE III (a). Electron micrograph of an air dried preparation of artificial top component. Notice how the particles have collapsed. Magnification × 90,000.

(b) Electron micrograph of a frozen dried preparation of artificial top component. Particles have retained structural integrity. Magnification × 90,000.



(a)

(b)

PLATE IV(a). Crystals of artificial top component. Phase contrast. Magnification × 2100.
(b) Crystals of artificial top component. Dark field. Magnification × 2500.

4. Discussion

Only a decade ago Markham put forward the hypothesis that TYMV was built up of an outer protein shell enclosing its relatively large amount of RNA. Markham based this on his discovery, in TYMV-diseased plants, of two types of particles with similar physico-chemical properties, but which differed in that only one of them was infectious and contained RNA. This concept has been accepted as a general principle governing the structure of smaller viruses.

In the case of TMV, knowledge has developed to the point where drawings can be made of the structure in which the RNA and the protein subunits fit in a helical pattern that outwardly results in a rod-like structure, with protein on the outside and RNA deeply embedded within.

In "spherical" viruses, and particularly in TYMV, evidence has accumulated that here too is a complicated geometrical structure composed of building units probably subdivided into an unknown number of chemical subunits of relatively low molecular weight. The arrangement of these subunits produces a spherical outward appearance (Klug & Finch, 1960). Although the evidence is scarce, it is assumed that—as is probably the case with TMV—this firm protein structure confers support to an otherwise relatively flexible chain of RNA.

The fact that a substance like artificial top component can be prepared in the manner described, with the particular sequence of events observed, has a few important implications for the problem of the structure of TYMV. Firstly, it provides additional evidence for Markham's basic hypothesis. Secondly, the structure of artificial top component must allow RNA fragments to leak out from within. It is possible that alkaline treatment damages the natural structure of the virus protein, although the damage cannot be gross as this would have been apparent in the physico-chemical properties of artificial top component. Another explanation would be that the spaces between subunits (Klug & Finch, 1960) extend right through the protein shell. Provided these holes were large enough, RNA material could leak out after becoming detached from the protein.

By reducing the time of treatment at pH 12 to as short as 15 sec, the conclusion is reached that the detachment of RNA is an all-or-nothing process. Either all of the RNA is removed or all of it remains within the protein shell, since only whole virus, artificial top component, RNA, and protein degradation products (longer treatments) appear in the reaction mixtures. No degradation product intermediate in size between virus and artificial top component could be detected. It seems likely that this means that the attachment of RNA to protein is accomplished largely by electrostatic interaction. Metal ions like Ca^{2+} and Mg^{2+} might mediate in these interactions, or might at least have a function in compensating excess negative charges.

Without further experimentation, no hypothesis regarding the possible presence of RNA subunits is proposed. Under the given experimental conditions with treatments of 35 sec, the released RNA has a sedimentation coefficient of about 8 s, which would roughly correspond to a molecular weight of 150,000 (Doty, Boedtker, Fresco, Hall & Haselkorn, 1959). This value for the sedimentation coefficient of the released RNA falls off to approximately 4 s when the treatment at pH 12 is extended to 30 to 60 min.

It has been reported that RNA with a sedimentation coefficient of 28 s can be extracted from TYMV (Cheng, 1959). This would fit in with the theory of a one-chain RNA molecule in TYMV with a molecular weight of about 2 million. If this is so,

then it seems that the 8 s RNA which is found on alkaline treatment merely represents polynucleotide chains formed by partial hydrolysis of the 28 s molecule. On the other hand, it must be borne in mind that the "flash" treatments at pH 12 are very mild indeed compared with the conditions under which alkaline hydrolysis is normally performed. After treatments of as long as 30 min at pH 12, the RNA has only degraded to 4 s material.

From purely geometrical considerations, and from their symmetry data on TYMV compared with top component, Klug & Finch (1960) have speculated on the presence of RNA subunits in TYMV. The experimental results quoted here are not inconsistent with the idea and the possibility is worth consideration. The subunits could be linked *via* bonds (other than covalent ones) which are easily broken by a short exposure to high pH conditions. Here too, a possible mediation through metal ions should be kept in mind.

It is planned to investigate this problem rather extensively in the near future.

So far, only the very first stages of the alkaline degradation have been thoroughly studied. Besides the complete protein shells, fragments of smaller size are found. In the ultracentrifuge, these protein fragments appear as a highly polydisperse system whose sedimentation rate decreases on more prolonged alkaline treatment. In electrophoresis, also, differences are found between material subjected to short or to long alkaline treatment. As this treatment is extended, the fragments appear as a slower migrating boundary. This could be interpreted as a slow dissociation of complete shells into smaller units, or aggregates of smaller units of varying size. Evidently the inter-subunit bonds are of a greatly different nature from those holding the RNA and protein together.

I wish to thank Professor H. Veldstra for his continuous interest and encouragement, and Miss C. Houwing for her capable assistance. The help of Mr. H. Heslinga, Mr. W. Soudijn and Mr. W. Godschalk at some stages of this investigation is gratefully acknowledged.

Thanks are due to Dr. R. L. Steere for doing the electron microscope work and to Dr. J. T. Finch and Dr. A. Klug for making the X-ray comparison of artificial and natural top component.

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X-ray "Powder" Diagrams of Crystals of an Artificial Top Component from Turnip Yellow Mosaic Virus

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X-ray powder diagrams have been taken of crystals of a high molecular weight protein component prepared by Kaper (1960*b*, foregoing paper) from turnip yellow mosaic virus by alkali degradation. The X-ray diagrams are indistinguishable from those of the natural, nucleic acid-free top component associated with the virus, and thus support the conclusion that Kaper's protein component corresponds to the intact protein shell of the virus devoid of RNA.

Very recently Kaper (1960*a*) has reported an alkaline degradation method for turnip yellow mosaic virus by means of which he has been able to prepare a protein component of sedimentation coefficient 53 to 54 s, which apparently corresponds to the intact protein shells of the virus devoid of RNA. This component therefore bears a strong resemblance to the natural component found accompanying the virus in infected plants (Markham, 1951) and, in the foregoing paper, Kaper (1960*b*) has made a comparison of a number of physico-chemical properties of the 54 s component with those of natural top component. In this note we describe the results of a limited X-ray crystallographic comparison of the two materials, which support the conclusion that this artificial top component may quite properly be identified with natural top component on structural grounds. This work was done on about 5 mg of the 54 s component kindly supplied by Dr. Kaper.

Crystals of the 54 s component can be grown from ammonium sulphate in just the same way as the virus and natural top component (Markham & Smith, 1949). Thus small crystals ($\sim 10\ \mu$) suitable for X-ray powder work were grown by adding a half volume of saturated ammonium sulphate to a solution of the 54 s component. Larger crystals ($\sim 100\ \mu$) were grown at room temperature in 0·9 to 1·0 M-ammonium sulphate, but slightly higher salt concentrations were required for crystal-growing at lower temperatures. These are exactly the same conditions of crystallization as those we have found for the virus and for natural top component. Furthermore, the crystals are identical in form with virus crystals, i.e. when symmetrically developed they are truncated octahedra characteristic of the cubic system, but they often show unequal development of the octahedral faces as can be seen in the photographs reproduced by Kaper.

For the X-ray work, we have taken photographs of a sludge of small crystals contained in a quartz capillary tube. Low-angle photographs were taken with CuKα radiation in a vacuum at a specimen to film distance of 20 cm, using a low-angle camera fitted with a bent-quartz focusing crystal. Higher-angle photographs were obtained in an evacuated Guinier camera of 12 cm diameter also using CuKα radiation focused from a bent-quartz crystal. Comparison photographs of a sludge of natural top component crystals were taken under exactly the same conditions.

The low-angle photographs show the crystal lattices of the 54 s particles and natural top component to be identical. The spacings and the intensities of the diffraction lines are the same. Natural top component scatters as a hollow spherical shell at low angles (spacings > 70 Å) (Schmidt, Kaesberg & Beeman, 1954), and the X-ray diagram shows a modulation in the intensities of the X-ray reflections corresponding to the first three maxima in the scattering curve of the equivalent spherical shell. An identical modulation occurs in the case of the 54 s crystals. The 220 reflection, which occurs at the spacing of first minimum of the scattering curve for the hollow shell and is thus absent for natural top component, is also absent in the case of the 54 s crystals.

The higher-angle photographs are identical up to spacings of at least 10 Å and, as far as we can tell, are also the same at higher angles where the scattering is relatively weak. (A photometer trace of the scattering from a sludge of crystals of natural top component has been published by Klug & Finch (1960).) However, this would not preclude small differences that would not show up in a photograph of an unoriented specimen. We hope in the future to compare single crystals of the two materials to investigate any fine differences that may be present.

The strong crystallographic similarity of the 54 s component to the natural top component leaves no doubt that they are essentially the same thing and, furthermore, implies that there has been no resultant denaturation or gross change in structure and configuration of the protein in the preparation of the artificial top component.

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Review Article

On Macromolecular Structure of Native High-Polymer Ribonucleic Acid in Solution

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This paper concerns some problems of the macromolecular structure of isolated high-polymer RNA in solution, and some new data are reviewed. The configurations of high-polymer RNA in solution, under different conditions, are discussed on the basis of available data on viscosity, sedimentation, light-scattering, optical rotation, u.v. absorption and electric dichroism measurements. Principally the infectious RNA of tobacco mosaic virus (TMV) is considered, as an object the native state of which may be judged by its biological activity.

The structure of high-polymer RNA in solution is presented from the point of view of several levels of organization: primary structure, secondary structure, tertiary structure.

The primary structure of the native infectious TMV-RNA is shown by physicochemical data to be one single continuous polynucleotide chain, with molecular weight of about 2×10^6. The continuity and the integrity of this chain is a decisive factor for the biological activity (the infectivity) of the RNA.

The principal feature of the secondary structure of native RNA in solution occurring in the presence of salts at room (or lower) temperature seems to be the presence of numerous, more or less short, helical chain regions stabilized by H-bonds. Their number depends upon the conditions of ionic strength, solvent composition, etc., and reversibly decreases with increase of temperature. In the absence of salts, or at high temperature, secondary structure is absent.

As to tertiary structure, the RNA in solution can be found in different states depending on conditions. In the complete absence of salts or at high temperatures the RNA molecule is an extended straightened polynucleotide chain or a very loose random coil. At room (or lower) temperature and low ionic strength the RNA molecule appears to be a compact rod formed by a linear piling of the successive and alternating helical and random regions of the chain, the short helical regions being arranged more or less regularly, and apparently tending to be perpendicular to the long axis of the particle. When the ionic strength increases, the compact RNA molecule transforms to a more coil-like state, and the orientation of the bases and the helical regions becomes less regular, the particle tending to be a compact coil with numerous helical regions within it.

High-polymer RNAs isolated from cellular organisms (bacteria, plants, animals) are apparently characterized by the same principal macrostructural features and the same structural transitions, depending on conditions of solution, as is the TMV-RNA. Data are presented which suggest that these RNAs are also single continuous polynucleotide chains and do not consist of shorter subunits held together by secondary bonds.

All isolated high-polymer RNAs in solution are considered to be analogous with respect to their macrostructure which seems to be typically a non-unique configuration arising principally from an equilibrium between the forces of H-bonding and electrostatic repulsion.

1. Introduction

The isolation of a biologically active, infectious RNA from tobacco mosaic virus (TMV) (Gierer & Schramm, 1956; Fraenkel-Conrat, Singer & Williams, 1957) demonstrated for the first time the possibility of preparing a free polymeric RNA known to be in a native state. This raised questions concerning the macromolecular structure of isolated native RNA in aqueous solution. At first, it seemed most likely that the native configuration of the isolated virus RNA in solution must be similar to the RNA chain configuration within the intact virus. Such a suggestion was voiced by Ginoza (1958). Later data, however, suggested a different conception of the state of the infectious RNA in solution. It was found that polynucleotide chains of the virus RNA in solution were in a much more random and more compact state than when within the virus particles (Gierer, 1958*a*; Boedtker, 1959; see also the earlier data of Hopkins & Sinsheimer, 1955). Moreover, the configuration of the infectious RNA in solution was shown not to be constant and stable, but to vary widely depending on ionic conditions (Boedtker, 1959; Spirin, Gavrilova & Belozersky, 1959; Haschemeyer, Singer & Fraenkel-Conrat, 1959; see also Hopkins & Sinsheimer, 1955). It became apparent that while the isolated RNA in solution lost the original configuration which had been inherent in the RNA within the virus, its biological activity was preserved.

At the same time it was shown that the problems of macromolecular structure of polymer RNA could not be solved by means of analogies with the structure of native DNA. It was found, simultaneously in a number of laboratories by means of different methods, that the RNA had no rigid two-stranded DNA-like structure, but was a flexible single polynucleotide chain (Gierer, 1957, 1958*a*; Ginoza, 1958, 1959; Hart, 1958; Spirin & Gavrilova, 1958; Boedtker, 1959; Spirin, Gavrilova & Belozersky, 1959). Analogous observations were also made in the case of non-viral polymer RNA (Eisenberg & Littauer, 1958; Littauer & Eisenberg, 1959).

Based on these data, the conclusion was drawn that the one-strand RNA chain in aqueous solution coiled into a random coil configuration, behaving as a typical contractile polyelectrolyte and possessing a great number of random intra-coil hydrogen bonds (Boedtker, 1959; Spirin, Gavrilova & Belozersky, 1959; Littauer & Eisenberg, 1959).

However, more recent observations have indicated that in buffer solutions at room temperature the macromolecular organization of the polymer RNA differs from that of a completely randomly coiled chain. Two independent communications have appeared which report a structural transition of the infectious virus RNA macromolecules upon heating of the solution. One, from Doty's laboratory, concerns the observation of intramolecular "helix-coil" transition, i.e. "melting" of intramolecular helical regions (Doty, Boedtker, Fresco, Haselkorn & Litt, 1959). Another, from our laboratory, concerned the finding of a transition of the whole coiled compact macromolecule to a more unfolded and open state, which occurred within a certain rather narrow temperature range and had the character of a phase transition (Gavrilova, Spirin & Belozersky, 1959).

In our later work, in collaboration with Professor Bresler's laboratory, this structural transition, occurring upon heating, was studied both from the viewpoint of intramolecular "helix-coil" transitions and from that of changes of the particle as a whole (Spirin, Gavrilova, Bresler & Mossevitsky, 1959).

The transition of the RNA particle as a whole was manifested by a sharp increase of specific viscosity occurring within a certain relatively narrow temperature range.

In the presence of urea, the specific viscosity increased at lower temperatures (about 10° to 20°C lower). Parallel studies of RNA sedimentation at high temperatures showed that the viscosity increase was accompanied by a drop in sedimentation coefficient of the infectious RNA. These viscosity and sedimentation changes indicated a sharp increase of the spatial dimensions of the RNA macromolecules, apparently resulting from the unfolding of polynucleotide chains upon heating. This was also supported by light scattering measurements at different temperatures. It was shown that the unfolded state of RNA macromolecules was characterized by the absence of configurational hypochromicity and of configurational optical rotation, i.e. by the absence of intramolecular H-bonding of bases. The changes mentioned were reversible and did not result in biological inactivation of the RNA. (Fig. 1, curve 1, shows an

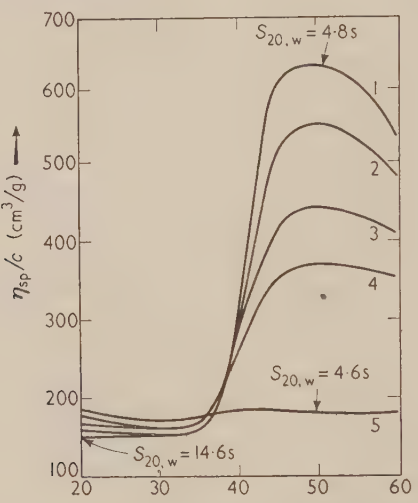

Fig. 1. Temperature dependence of reduced viscosity of virus RNA solution; phosphate buffer pH 7, ionic strength 0·1, in the presence of 6 M-urea; RNA concentration is 0·36% ($3{\cdot}6 \times 10^{-3}$ g/cm^3).

1, the freshly prepared infectious RNA.

2–4, the same RNA exhibiting progressively decreasing infectious activity, as a result of incubation of the solution at room temperature.

5, the same RNA exhibiting no activity, as a result of incubation of the solutions (spontaneous inactivation).

experiment on the measurement of the viscosity and sedimentation temperature dependence of freshly-prepared virus RNA; ionic strength 0·1, urea concentration 6 M, RNA concentration $3{\cdot}6 \times 10^{-3}$ g/cm^3.) Decrease of the ionic strength of the solvent or decrease of RNA concentration in the solutions (i.e. decrease of the concentration of counter-ions) was shown to result in a lowering of transition temperature and in a broadening of the transition temperature range. Increase of ionic strength acted in the opposite direction.

In connection with these data on the structural transition of virus RNA macromolecules in solution upon heating, reference should be made to a communication of Fraenkel-Conrat's group concerning two configurations of TMV-RNA (Fraenkel-Conrat, 1959; Haschemeyer *et al.*, 1959). They reported that RNA, in water in the absence of salts, could be characterized by two states of its macromolecules, depending on the presence or absence of RNA-bound (RNA-chelated) cations of bi- and polyvalent metals. In the presence of such counter-ions in the preparations, the RNA

macromolecules were reported to be in a compact coiled state and to contain ordered (helical) regions; in the absence of those cations, the macromolecules apparently were in a more or less unfolded, loosened, and random state.

Also, Cox & Littauer (1959) have reported a structural transition of polymer RNA macromolecules from *E. coli* resulting from a lowering of ionic strength. This transition was manifested by a sharp increase of viscosity and a decrease of sedimentation coefficient, just as was the case in our experiments. Thus, they supported Fraenkel-Conrat's and our data on two configurations of RNA; they showed, by another method and with other material, that the transition from the compact configuration to the unfolded one bore the marks of a phase transition.

On the basis then of the data obtained up to the present time a certain picture of the macromolecular structure of native high-polymer RNA in solution (in particular of the infective TMV-RNA) may be formed. The available data allow one to think that the principal forces determining the configuration of the RNA macromolecules in solution are: (*a*) H-bonding of the bases of the flexible and coiled polyribonucleotide chain; (*b*) electrostatic repulsion of the phosphate groups of the nucleotide residues along the chain. This postulate does not rule out the possibility that internal linkages formed by metal-complexes (of the type of phosphate-metal-phosphate or purine-metal-phosphate bonding) may also play an essential role in the formation of the compact structure of RNA. The complete reversibility of the structural transition suggests that the configuration of the RNA macromolecule in solution is a consequence of an equilibrium of the molecular forces mentioned; the structure seems to be devoid of any unique and fixed spatial inter-relations between various regions and parts of the chain and to possess a structure which is governed by the free and reversible interaction of various regions and parts of chain in solution. At the same time, this interaction results in the existence of a certain order in the macromolecule and of a certain compact packing of the chain as a whole.

For the characterization of the macromolecular structure of RNA, it appears to be helpful to use the terminology which is accepted at the present time for the definition of the macromolecular organization of polypeptides and proteins. According to this terminology, macromolecular structure is considered from the point of view of several levels of organization: primary structure, secondary structure, tertiary structure.

Considering the macromolecular structure of native RNA in solution, it is the infectious TMV-RNA that will be kept in mind in the first place.

2. Primary Structure

All available evidence indicates that the molecular weight of the native virus RNA is about 2×10^6 and that a particle of this size is necessary for biological activity. However, the problem whether the polynucleotide chain in the macromolecule is continuous or interrupted is not solved by these data. The question is whether the whole high-molecular RNA chain is a single continuous polynucleotide chain, or whether such RNA consists of several shorter polynucleotide chains, or subunits, held together by secondary bonds.

In our laboratory an attempt was made to answer this question on the basis of physico-chemical data, after succeeding in finding a method of "unfolding" the RNA macromolecule (Gavrilova *et al.*, 1959). It was found that the RNA particles retained their high molecular weight at high temperature, and did not undergo dissociation as a result of the breaking of the secondary bonds in RNA macromolecules and of the unfolding of the RNA chains. The average molecular weight of the infective RNA

preparations in the state of unfolded chain configuration was $1{\cdot}5 \times 10^6$, according to light-scattering data (Spirin, Gavrilova, Bresler & Mossevitsky, 1959). These data, therefore, suggested that the primary structure of the infectious RNA macromolecules is one single continuous polynucleotide chain, and not an association of shorter chains or polynucleotide subunits.

Quite a different picture was shown by virus RNA after spontaneous loss of its infectivity, a result of keeping the RNA solution for a long period of time. It was observed in a number of cases that this inactivated RNA did not differ from an infectious preparation either in magnitude of specific viscosity or in the average size of the particles at room temperature (Gavrilova & Spirin, 1959). Important differences between the infectious RNA preparations and the spontaneously inactivated ones were found however when the temperature of solution was raised up to that of the structural transition (Gavrilova *et al.*, 1959). As has already been pointed out above, on heating of the solution the infectious macromolecules extended into long chains with a resultant increase of the viscosity and a drop of sedimentation coefficient. Under these conditions the uninfectious (inactivated) particles were found to dissociate as a result of chain extension to shorter chains, exhibiting no increase of viscosity (Fig. 1, curve 5). From this result the conclusion was drawn that the continuous structure of the native RNA transformed into an interrupted RNA structure during the spontaneous inactivation.

Following this discovery, it was possible to develop a correlation between the magnitude of the viscosity increase upon heating and the degree of the infectivity of the RNA solution during the process of the spontaneous inactivation. A decrease of the magnitude of the temperature rise of the specific viscosity proceeded with the spontaneous inactivation (Fig. 1). This fact could be used to provide a simple physicochemical criterion of the biological activity of the virus RNA preparations. This phenomenon, the increase of viscosity upon heating (as well as magnitude of the specific viscosity at the appropriate temperature), seems to be a good measure of the true length of the high-polymer chain and a test for the intactness of the primary structure of RNA macromolecules.

3. Secondary Structure

The native infectious TMV-RNA was reported by Gierer to possess rather significant optical activity in buffer solution while the constituent nucleotides as such, or even a ribonuclease digest of the RNA studied, were characterized by a small or negligible optical activity (Gierer, 1957, 1958*a*). Later, the optical activity of the native infectious RNA in buffer solutions was shown to bear a direct relation to hydrogen bonds within the RNA macromolecule (Doty *et al.*, 1959; Spirin, Gavrilova, Bresler & Mossevitsky, 1959). Hydrogen bonds of RNA were found to be, in large part, not randomly arranged, but such as to create a certain structure in the form of helical regions within the macromolecule. The optical activity by itself, found formerly by Gierer, was shown to be none other than the result of these helices stabilized by the hydrogen bonds. Thus, the infectious RNA macromolecules in buffer solutions are characterized by a certain secondary structure.

Doty *et al.* (1959) presented data that about 40% of nucleotides of the RNA chain, in buffer solutions at room temperature, were organized in helical regions stabilized by hydrogen bonds. The remaining part of the chain has no secondary structure and is non-organized. According to Doty *et al.*, the principal features of the helical regions are the following: (1) the helical regions of the macromolecule consist of two strands

running in opposite directions and being parts of the same RNA chain; these parts come in contact owing to bending and looping within non-helical regions; (2) the helical regions are relatively short and seem to be imperfect and to include some defects (unmatched bases).

4. Tertiary Structure

The macromolecular chain of the native (virus) RNA in solution is coiled in asymmetrical particles with molecular weight of about 2×10^6. One may ask: How are the polynucleotide chain and its helical regions packed in this particle? Are the helical regions arranged at random inside the particle, or are they oriented in some manner with respect to the long axis? What is the RNA particle like in solution—is it a rigid rod with quite a definite ordered arrangement of the chain and orientation of the helical regions, or is it a compact asymmetrical coil with random distribution of the helical regions and random arrangement of the chain?

It is evident that the RNA can be found in different states depending on conditions.

(a) It is rather obvious that when the RNA is in water in the absence of RNA-bound bivalent cations (Haschemeyer *et al.*, 1959), or in buffer solutions at high temperatures (Gavrilova *et al.*, 1959; Spirin, Gavrilova, Bresler & Mossevitsky, 1959) it exists in more or less unfolded configuration, as a single strand which may be extended in length to a very considerable degree. Thus, under these conditions, the RNA macromolecule as a whole is an extended, straightened polynucleotide chain or a very loose random coil.

(b) In contrast to this, a very compact coiled configuration of RNA is observed in buffer solutions, at room temperature or at 5°C (Gierer, 1958*a*; Boedtker, 1959; Spirin, Gavrilova, Bresler & Mossevitsky, 1959). RNA in water can be not only in the unfolded (extended) configuration, but also in a compact (coiled) arrangement as well, if the molecule itself contains the RNA-bound cations of bi- and poly-valent metals (Haschemeyer *et al.*, 1959). It is this compact configuration that is characterized by the presence of helical regions, i.e. by a secondary structure. Transition from this configuration to an unfolded one is not completely gradual but bears the character of a phase transition, or "melting" (Doty *et al.*, 1959; Gavrilova *et al.*, 1959; Spirin, Gavrilova Bresler, & Mossevitsky, 1959). The unfolded configuration and the coiled one are apparently two distinct qualitatively different states of RNA chains.

Considering earlier data on the high-polymer virus RNA such as those of Hopkins & Sinsheimer (1955), Hart (1955, 1958), or of Ginoza & Norman (1957), one can see that it is the compact (coiled) configuration, with which these authors have dealt when studying the RNA in water. The measured RNA particle lengths were 1600 Å or 3000 Å respectively. At the same time, the data of Hopkins & Sinsheimer on light scattering of the virus RNA solutions indicated a rod-like model of RNA macromolecules in water at very low ionic strengths. The electron microscope observations of Hart showed directly that the RNA was in the form of rods with length about 3000 Å and diameter (in the dry state) about 30 to 40 Å when samples were prepared from RNA solution in water. Thus, there seem to be grounds for believing that the RNA macromolecule as a whole can form a more or less rigid rod, either when it is at room temperature in solutions of low ionic strength or in water in the presence of RNA-bound bivalent cations.

Further, on the basis of preliminary electric birefringence measurements of the virus RNA solutions in water, Ginoza & Norman (1957) noted that the magnitude of the birefringence was such as to suggest a highly ordered structure. The sign of the

birefringence was positive, in contrast to the negative sign observed with native DNA. The authors considered this fact to indicate that the bases were parallel to the long axis of the RNA molecule. Unfortunately, the authors do not give any details of the birefringence measurements so that it is impossible to analyse their data as such. In any case, a better answer to the question of the base orientation within the molecules can be obtained from measurements of the ultraviolet dichroism of RNA solutions, than from birefringence measurements.

Recently Dvorkin & Spirin (1960) carried out electric ultraviolet dichroism measurements of infectious virus RNA solutions, by means of a special technique worked out by Dvorkin (1960). At room temperature and low ionic strength the ultraviolet absorption, when the electric field vector was perpendicular to the plane of light polarization, was shown to be appreciably lower than in the case when the electric field vector was in the plane of light polarization. In other words, the sign of the ultraviolet dichroism of the RNA was positive, and opposite to that of native DNA. The dichroic ratio at 260 mμ in water or in a solution of ionic strength 0·01, at an electric field gradient of 120 v/cm and higher, was shown to be about + 1·2. These results prove that the RNA macromolecule, under the conditions considered at any rate, is not a random coil. The coiled RNA configuration, in solutions of low ionic strengths at room temperature, thus appears to be characterized by a more or less ordered arrangement of the chain within the particle. The positive sign of ultraviolet dichroism actually appears to indicate a mainly parallel orientation of the planes of the base rings with respect to the long axis of the compact RNA particle.

The base rings in polynucleotides are known to have an arrangement near to perpendicular with respect to the polynucleotide chain. Consequently, a principally parallel orientation of the bases with respect to the long axis of the macromolecule suggests that the directions of polynucleotide chains within the coiled RNA macromolecule have a tendency to be perpendicular to the long axis of the macromolecule. The irregular regions of the RNA chain could hardly be of such a definite orientation. The most probable situation is that there are short helical regions of the chain which are arranged in a regular manner within the RNA macromolecule. A rod-like model of the RNA molecule can be preserved if the short helical two-stranded regions are arranged mainly perpendicularly to the long axis of the particle. A compact rod can be formed, for example, by piling of the successive and alternating helical and random regions of the chain as may be seen in Fig. 2(b).

(c) When the ionic strength of the solution is reduced, the RNA macromolecule has been shown to have a tendency to reduce its length (Hopkins & Sinsheimer, 1955; Boedtker, 1959). Simultaneously an additional number of intramolecular hydrogen bonds is formed (Boedtker, 1959; Spirin, Gavrilova & Belozersky, 1959). In this condition, the transition from the compact coiled configuration to the loose unfolded one, occurring upon heating, is not completely correlated with the "melting" of the helical regions and the rupture of hydrogen bonds; instead this transition is more abrupt and shifts to the higher temperature part of the "melting" temperature range (Spirin, Gavrilova, Bresler & Mossevitsky, 1959). This result would seem to indicate that at sufficient ionic strength (0·1 and higher) "melting" of the helical regions within the macromolecule is not immediately reflected in the whole RNA configuration and does not immediately result in unfolding: melting of the first portion of helical regions does not result in the corresponding lengthening (extension) of the molecule.

This might be expected to be the case if individual parts and folds of the chain were held together, in a coiled state, by links of more than one double-helical region. If

this is the case, the data presented concerning the properties of RNA at sufficiently high ionic strength must indicate that the above-mentioned rod-like compact macromolecule will tend to further folding and coiling, with the formation of additional helical links between different parts of the chain when the ionic strength of the solution is increased. In other words, an increase of the ionic strength seems to result in a transition to a more coil-like state, the coil being very compact and containing a great number of helical regions.

This supposition is supported by ultraviolet dichroism measurements of RNA solutions at high ionic strength at 20°C. The magnitude of the dichroic ratio is reduced to about 1·1 when the ionic strength is increased to 0·1 (Dvorkin & Spirin, 1960) which appears to indicate a more random arrangement of the helical regions within the particle at the higher ionic strength.

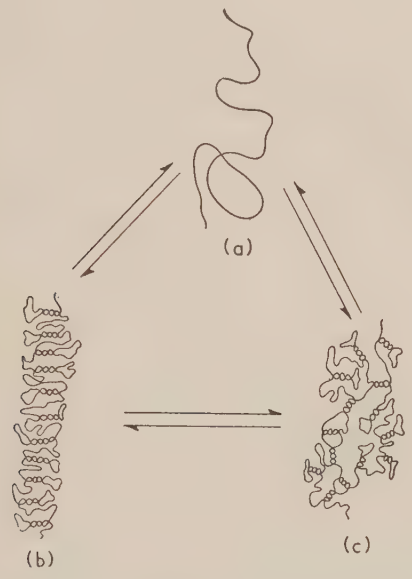

Fig. 2. Possible configurations and structural transitions of the high-polymer RNA macromolecule in solution, depending on ionic strength and temperature.
(a) Loose random coil or unfolded chain (in the absence of salts or at high temperatures).
(b) Compact rod, with helical regions arranged more or less regularly (in low ionic strength at low temperatures).
(c) Compact coil, with helical regions (in high ionic strength at low temperatures).
a $\rightleftharpoons$ b Addition of a salt to a saltless aqueous solution, and, reversibly, removal of salt, at low temperatures (i.e. room temperature or below); or heating of the solution (b $\rightarrow$ a) and, reversibly, cooling of the heated solution, at low ionic strengths.
b $\rightleftharpoons$ c Increase, and, reversibly, decrease of ionic strength, at low temperatures (i.e. room temperature or below).
c $\rightleftharpoons$ a Heating of the solution and, reversibly, cooling of heated solution, at high ionic strength.

All of the cases considered (a, b, c) and the transitions of the RNA configurations in solution are presented, very tentatively and schematically, in Fig. 2.

The most recent data on the temperature dependence of the ultraviolet dichroism of virus RNA solutions in an electric field (Dvorkin & Spirin, 1960) lend support to this general scheme of transitions. The positive dichroism of the virus RNA solution at low ionic strength (0·01) was characterized by the same magnitude of the dichroic ratio, D, + 1·2, at 10°, 20° and 30°C. The dichroism disappeared, however,

at 40°C ($D = 1$). At 50°C the sign of the dichroism became negative, the dichroic ratio (D) being about — 1·1. The inversion of the sign of the dichroism upon heating was reversible. The stated changes, occurring in parallel with the transition from the compact configuration to the unfolded one, seem to prove that the unfolding of the chain is actually accompanied by a change of prevalent orientation of the bases, from one parallel to the long axis of the molecule when the chain is arranged perpendicularly to this axis, to one perpendicular to the long axis when the chain is extended and straightened. At higher ionic strengths (0·1), a similar pattern of inversion of the sign of the dichroism was also observed upon heating, but the absolute magnitudes of the dichroic ratios were relatively smaller, indicating, apparently, more coil-like configurations.

In conclusion, it should be stressed once again that all the changes and the structural transitions discussed, involving secondary and tertiary structure of the virus RNA in solution, are reversible and do not result in biological inactivation of the RNA. Intactness and the unique specificity of the polynucleotide chain itself, i.e. of the primary structure, are important for biological activity (infectivity), while one or another configuration of this chain in the inoculated solution seems to be of no decisive importance.

5. On the Structure of High-Polymer RNA from Cells

High-polymer RNAs have been obtained not only from viruses but from bacterial cells (Littauer & Eisenberg, 1958, 1959), plant tissues (Gierer, 1958*b*) and animal tissues (Gierer, 1958*b*; Laskov, Margoliash, Littauer & Eisenberg, 1959; Hall & Doty, 1959) including ascites cells (Timasheff, Brown, Colter & Davies, 1958) as well. Apparently, these high-molecular RNAs with molecular weights of the order of 10^6 are constituents of the ribosomes. The phenol method used in all these investigations is believed to preserve the RNA isolated in its native state. Studies on these RNA preparations in solution show that they are analogous to the infectious virus RNA in their physico-chemical properties. All of the works mentioned state that the cellular RNAs in salt or buffer solutions are compact coiled asymmetric particles. These particles change their shape and size reversibly, depending on ionic strength. They show exactly the same kinetics of ribonuclease break-down as does the virus RNA in solution. The ratios of the values of molecular weight, of sedimentation coefficient, and of specific viscosity also coincide completely with those in the case of TMV-RNA. These cellular RNAs in solution show the same magnitude of hypochromicity, the same proportion of hydrogen bonds within the macromolecules, and the same dynamics and character of their reversible changes under pH, ionic strength and temperature action as does the virus RNA. Finally, a structural transition of cellular RNA macromolecules on lowering the ionic strength has been reported (Cox & Littauer, 1959). The problem of the structural transition and unfolding of the whole RNA chain under heating was studied by Hall & Doty (1959) with ribosomal RNA. Contrary to our data on virus RNA (Gavrilova *et al.*, 1959; Spirin, Gavrilova, Bresler & Mossevitsky, 1959) they found that dissociation of the RNA particles into shorter chains occurred upon heating the ribosomal RNA solution, accompanied by a reduction of the specific viscosity (Hall & Doty, 1959).

It is very likely, however, that this phenomenon is a consequence of some secondary damage to the integrity of the ribosomal RNA chain which might take place during the isolation of the ribosomes, and does not reflect the native state of the polymer RNA

at all. Recently Spirin & Milman (1960) studied the temperature dependence of the specific viscosity of RNA preparations isolated directly from rat liver and ascites tumor cells by the phenol method. The low-molecular fractions were separated by means of the precipitation of the RNA with 1 M-NaCl. It was found that this polymeric RNA from animal cells did exhibit a sharp increase—a jump—of viscosity upon heating just as the virus RNA did; this increase occurred within approximately the same temperature range as that of TMV-RNA. This result strongly suggests that the native polymeric cellular RNA macromolecule does not consist of shorter subunits but is one single continuous chain, just as is the virus RNA.

This leads us to conclude that the macromolecular structure of high-polymer RNA isolated from cellular organisms, is, in solution, analogous to that of native virus RNA under the same conditions. On these grounds it is possible to think that free proteinless high-molecular RNAs in solution are, in general, analogous and that their macrostructure in solution is the typical equilibrium configuration of one single continuous polynucleotide chain; this configuration possesses the same main structural features discussed above, in the case of the native (infectious) virus RNA in solution.

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Letter to the Editor

Structure of Ribosomes from *Escherichia coli* as revealed by their Disintegration

Ribonucleoprotein (RNP) particles are found in living cells and are known to be vitally involved in the synthesis of protein molecules. Although the intact particles have been repeatedly observed in the electron microscope (Hall & Slayter, 1959; Huxley & Zubay, 1960) virtually nothing is known about their internal organization. In the hope of discovering something of their architecture we have followed with the electron microscope the breakdown of RNP particles from *E. coli*.

RNP particles were obtained from exponentially growing cultures of *E. coli* as described by Bolton, Britten, Cowie, McCarthy, McQuillen & Roberts (1959). The particles were suspended in a buffer (TSM) containing 0·01 M-tris, 0·02 M-succinic acid, 0·01 M-magnesium acetate, with pH adjusted to 7·6. Under these conditions the RNP particles are known to be aggregated in the form which has a sedimentation coefficient of 100 S (Tissières, Watson, Schlessinger & Hollingworth, 1959; Britten & McCarthy, 1959).

Breakdown of the particles was effected by removal of magnesium. Using the method of Elson (1959) it was shown that when this is done the RNA is made acid-soluble, presumably by the now activated RNase enzyme known to be present in latent form in the intact particle (Elson, 1959; Bolton & McCarthy, 1959). Figure 1 shows the progress of the reaction for a 0·1% suspension of RNP particles in 0·1 M-EDTA (ethylenediaminetetra-acetic acid) at pH 7·6. It is seen that the reaction is largely completed in two hours. During this period 90 ± 10% of the RNA is rendered acid-soluble.

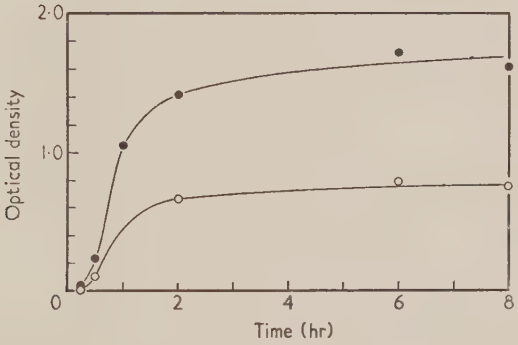

Fig. 1. Rate of production of acid-soluble RNA from 0·1% suspension of RNP particles in 0·1 M-EDTA, at pH 7·6. ● 260 mμ; ○ 280mμ.

For electron microscopy single drops (about 0·1 ml.) of 5×10^{-4} % suspensions of RNP particles in TSM were placed on formvar films which floated on 50 ml. of water or 0·1 M-EDTA solution at pH 7·6. In some experiments EDTA was added to the suspension of RNP particles. The liquid beneath the film was periodically pipetted out and replaced. At the end of the dialysis, the films with the drops were picked up on copper grids, air-dried, shadowed with platinum at an angle of 4 to 1 and examined

in the electron microscope. Varying degrees of degradation were obtained by stopping dialysis after different lengths of time.

After 2 hr of dialysis against each of 3 changes of distilled water the majority of particles appear elongated and consist of 2 portions. Their long and short dimensions are approximately 250 Å and 180 Å. The two components are clearly visible at A in Plate I(a). The doublet character observed here is consistent with that of the 70 s particle which consists of one 30 s and one 50 s. The dimensions given by Hall & Slayter (1959) for this are 200 by 170 Å. The coarse background at this stage of dialysis indicates that all the salt has not yet been removed. Thus after this dialysis the 100 s particles have broken down to 70 s particles but not further.

After 24 hr of dialysis with 4 changes of distilled water the early stages of disintegration of the particles are visible. The doublet 70 s components have separated into the rather spherical 50 s component (A in Plate I(b)) and the more asymmetric 30 s component (B in Plate I(b)). This separation is to be expected on removal of magnesium. In addition many of the particles have short fibrils attached to them (C in Plate I(b)). The thickness of these fibrils is very roughly 30 Å. They presumably represent material released from the particles. This fibrillar material which is seen emerging from the particles may well be the RNA loosened by removal of the magnesium ions. The 30 Å estimated diameter of these fibrils is too great for single-stranded RNA and probably even for double-stranded RNA. Conceivably these strands also carry attached protein.

Occasionally one sees the 50 s and 30 s particles a small distance from each other but joined by the fibril (D in Plate I(b)). This suggests that in some of the 70 s particles, the 30 s member is so tightly bound to the fibrillar component of the 50 s particle that the fibril is released from the 50 s particle before the 30 s breaks away. It is interesting in this connection that Tissières, Schlessinger & Gros (1960) have described a fraction of the 70 s particle which is relatively resistant to breakdown on removal of magnesium. This fraction is responsible for essentially all the *in vitro* incorporation of amino acids.

More complete removal of magnesium was effected by dialysing against EDTA for 3 hr, then twice against water for 2 hr. Adding EDTA to the suspension and dialysing it away after 2 hr led to similar results. Plate I(c) shows a typical area of the specimen. The structures observed here were similar to those found after very long dialysis against water. A large number of cup-like hollow structures are visible. They are indicated by arrows in Plate I(c). Their diameter is somewhat variable between 200 Å and 400 Å. The central hollow has a diameter varying from "hardly visible" to about 200 Å. The structures with larger external diameters also have larger hollows suggesting that some of the structures are loosened more than others. The size and shape of these structures compare with those of the 50 s particles suggesting that they are derived from them.

RNP particles are known to consist of about 60% RNA and 40% protein. Our chemical evidence cited earlier indicates that the breakdown involves a removal and degradation of essentially all the RNA component. The hollow cup-shaped structures which remain after degradation must then be the protein components. The view that the RNA of the RNP particles is embedded in protein is consistent with the observations of Elson (1959) and of Tissières *et al.* (1959) that the particles are relatively resistant to RNase.

The walls of the cup-shaped structures are not smooth. Particularly where the degradation has been extensive and the structure is loosened (A in Plate I(c)), it is

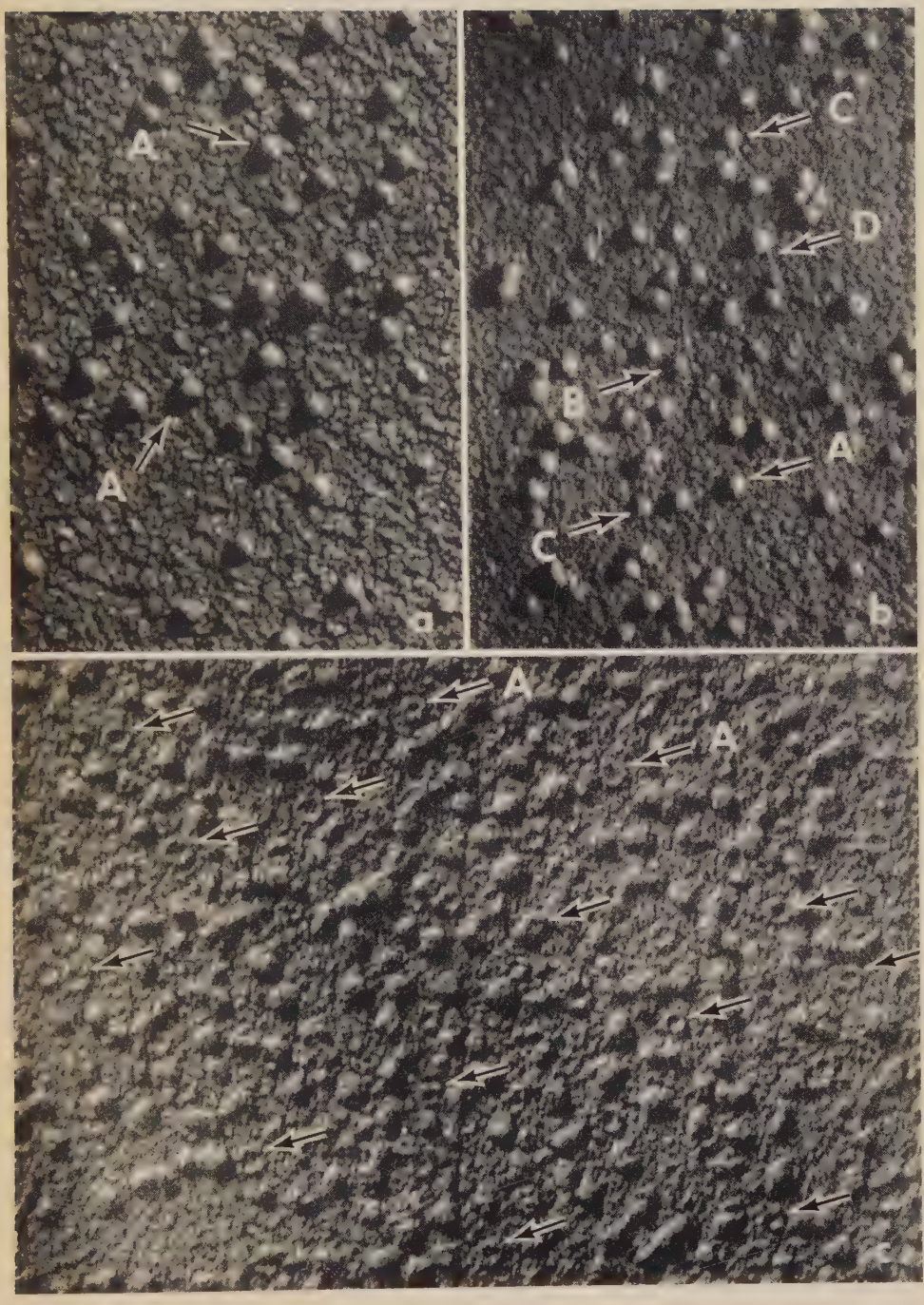

PLATE I(a). Electron micrograph of RNP particles after short dialysis from TSM buffer into distilled water. Most of the particles are the 70 s doublets. The components of the doublets are readily seen at A. Magnification × 120,000.

(b) Electron micrograph of RNP particles after long dialysis from TSM buffer into distilled water. 50 s particles are visible at A and 30 s particles at B. Fibrils being released by the 50 s components can be seen at C. The 50 s and 30 s particles slightly separated and joined by a fibril are shown at D. Magnification × 120,000.

(c) Electron micrograph of RNP particles after dialysis from TSM buffer into EDTA followed by distilled water. The hollow cup-like structures shown by the arrows are believed to be the protein shells which remain after the RNA has become digested. At A the structure has been loosened considerably and the globular components are readily visible. Magnification × 120,000.

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possible to discern components of the wall which are globular and which have a diameter about 20 to 50 Å.

After degradation of the particles, our micrographs showed no structures which could be clearly related to the 30 s particles. Experiments using purified particles are now in progress to discover their fate.

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